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# *Canadian Journal of Microbiology*

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## CORRECTIONS

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Page 656. Equation 3 should read

$$K = \frac{k_b T}{h} e^{\Delta S/R} - e^{-\Delta H/RT}.$$

Page 659. In Table II, $E$ values for $K_0$ should all be minus quantities, i.e. $-4{,}000$; $-3{,}500$; and $-3{,}800$ cal/mole.

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# THE RAPID PRODUCTION OF ANTISERA AGAINST ATTENUATED POLIOVIRUSES[1]

R. C. FRENCH, P. MOREAU, AND W. YAROSH

## Abstract

Culture fluids obtained after infection of human kidney cells with Sabin's attenuated strains of polioviruses contained $10^7$ to $10^8$ plaque-forming units per milliliter. When four intravenous injections of such fluids were administered during 6 weeks to rabbits or roosters, the antisera obtained were capable of neutralizing equal volumes of undiluted vaccines.

## Introduction

Live poliovirus vaccine preparations for oral use are currently produced by propagating selected attenuated strains in monkey kidney tissue culture under rigidly controlled conditions. One of the more important tests (5) employed for the detection of undesirable extraneous viruses in the vaccine depends upon neutralization of the poliovirus by specific antisera, and the subsequent inoculation of such serum–virus mixtures into tissue cultures of 'normal' monkey kidney. Previous reports (3, 4) and methods in current use require from 3 to 9 months to produce high titer rabbit antisera, despite the use of virulent polioviruses as antigens. A preferable method, described in this work, utilizes attenuated polioviruses propagated in human cells and requires only 7 weeks to produce antisera of sufficiently high titers for the above test purposes.

## Materials and Methods

In addition to the virulent strains of Mahoney, MEF1, and Saukett polioviruses used routinely in our laboratories, the following attenuated strains were obtained through the courtesy of Dr. A. B. Sabin: type I, LS*c*,2*ab*; type II, P712,*ch*,2*ab*; and type III, Leon, 12$a_1b$. Cultures of HeLa and human kidney (HK/55) cell lines were obtained from Dr. S. F. Kitchen of these laboratories and of human amnion (FL) from Dr. J. Fogh. All cell lines had been passaged at least 40 times in our laboratory, medium M 150 containing 5% or 10% calf serum being used for the maintenance or outgrowth of cells,

[1]Manuscript received July 5, 1960.
Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.



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respectively. The same medium containing only 2% calf serum was used for the outgrowth of primary monkey kidney cells. The general methods employed, including those for the assay of plaque-forming units (pfu) and for the determination of plaque-neutralizing titers of antisera have been described elsewhere (1).

The attenuated virus suspensions used as antigens were propagated in human kidney cells and are described later. At the commencement of immunization the New Zealand white rabbits averaged 3.8 kg and the white Leghorn roosters were from 6- to 12-months old. All injections were administered intravenously, doses of 2 ml being given to rabbits and of 1 ml to roosters. The immunization schedule was based upon the experience previously gained with roosters by Furesz (2). A primary course of three injections, spaced at weekly intervals, was administered to each animal. One week after the third injection each was 'trial' bled ("A" serum). Three weeks later a first booster injection was given, followed by a trial bleed 1 week later ("B" serum). After a further interval of 3 weeks, a second booster injection was administered and 1 week later all animals were exsanguinated ("C" serum).

All blood samples from rabbits were received into dry containers and the sera later separated by centrifugation and heated at 56° C for 30 minutes. Roosters were bled into a citrate solution and after removal of red cells the plasma samples were similarly heated. After storage at 4° C for 2 days fibrin was removed by sedimentation.

## Results

*Growth of Attenuated Polioviruses in Human Cells*

Preliminary experiments indicated that Sabin's attenuated strains of polioviruses propagated readily in all three human cell lines at 36° C. Accordingly, monolayer cultures in Roux bottles of HeLa and human kidney cells which had grown out until confluent had their medium decanted at 8 days and were then each washed twice with 50-ml amounts of M 150. Each bottle then received 50 ml of serum-free medium containing a 1/300 dilution of Sabin's seed virus, two Roux bottle cultures of each cell line being inoculated with each type of virus and incubated at 36° C. At 3 days and 5 days after inoculation, one culture of each pair was harvested aseptically and cellular

TABLE I

Growth of attenuated* polioviruses in human cells

| Cell line | Days at 36° C | $Log_{10}$ pfu per ml | | |
|---|---|---|---|---|
| | | I | II | III |
| HeLa | 3 | 7.65 | 6.88 | 7.16 |
| | 5 | 7.88 | 7.33 | 7.84 |
| Human kidney | 3 | 8.32 | 7.08 | 7.90 |
| | 5 | 7.75 | 7.52 | 7.55 |

*Type I, LS*c*,2*ab*; type II, P712,*ch*,2*ab*; type III, Leon, 12*a*$_1$*b*.

debris removed at 4° C by sedimentation for 15 minutes at 1800 r.p.m. The resulting virus suspensions were assayed for pfu with the results shown in Table I.

*Antigenic Responses in Rabbits and Roosters*

The attenuated virus suspensions used as antigens consisted of pools of the materials grown in human kidney cells and harvested at 3 and 5 days after infection (Table I), and contained approximately $10^{8.0}$, $10^{7.3}$, and $10^{7.7}$ pfu per ml for types I, II, and III, respectively.

TABLE II

Antigenic response of rabbits to attenuated polioviruses

| Virus type | Rabbit | Plaque-neutralizing titer* of serum | | |
|---|---|---|---|---|
| | | "A" serum† | "B" serum | "C" serum |
| I | 81 | 2,000 | 7,500 | 12,000 |
| | 83 | 1,200 | 2,000 | 2,000 |
| | 84 | 4,800 | 22,000 | 22,000 |
| | 85 | 6,000 | 14,000 | 12,000 |
| | Pooled serum | 3,500 | 11,000 | 12,000 |
| II | 87 | 2,500 | 5,000 | 8,300 |
| | 88 | 1,100 | 9,000 | 6,300 |
| | 89 | 500 | 600 | 1,000 |
| | Pooled serum | 1,400 | 4,000 | 5,300 |
| III | 90 | 1,800 | 1,400 | 1,200 |
| | 91 | 2,200 | 3,000 | 900 |
| | 92 | 5,800 | 6,800 | 6,200 |
| | 98 | 50 | 1,000 | 500 |
| | Pooled serum | 2,400 | 3,000 | 2,200 |

*The reciprocal of the serum dilution which neutralized 50% of the virus under standard test conditions.
†The A, B, and C sera were taken 1 week after the third injection, the first booster dose, and the second booster dose, respectively.

TABLE III

Antigenic response of roosters to attenuated polioviruses

| Virus type | Rooster | Plaque-neutralizing titer of plasma | | |
|---|---|---|---|---|
| | | "A" plasma | "B" plasma | "C" plasma |
| I | 151 | 1,800 | 6,000 | 3,000 |
| | 152 | 2,500 | 60,000 | 30,000 |
| | 153 | 2,000 | 12,000 | 16,000 |
| | 155 | 500 | 2,000 | 3,000 |
| | Pooled plasma | 1,700 | 21,000 | 13,000 |
| II | 157 | 2,100 | 18,000 | 8,200 |
| | 158 | 700 | 5,700 | 3,000 |
| | 159 | 200 | 6,300 | 3,800 |
| | Pooled plasma | 1,000 | 10,000 | 5,000 |
| III | 160 | — | 24,000 | 29,000 |
| | 162 | — | 40,000 | 16,000 |
| | 163 | — | 2,100 | 3,500 |
| | Pooled plasma | 2,000 | 22,000 | 17,000 |

The plaque-neutralizing (PN) titers of the individual and pooled sera, listed in Tables II and III, represent the reciprocals of the serum dilutions which, when mixed with equal volumes of virulent polioviruses containing 1000 pfu per ml and incubated for 3 hours at 37° C, were found to neutralize 50% of the virus. All titers listed are the averages of at least two titrations and are of the same order as those obtained in the more conventional test, where such serum–virus mixtures are inoculated into tube cultures of monkey kidney cells which are then observed microscopically during the ensuing week for cytopathic effects (CPE). Five of the pooled "A" sera listed in Tables II and III were titrated by both methods and it was found that the PN titers were on the average 30% higher than the CPE titers.

*Neutralization of Attenuated Polioviruses*

The antisera were next tested for their ability to neutralize the polioviruses present in undiluted preparations of oral vaccines. For this purpose the individual rabbit "C" sera were pooled according to type and the relatively small amounts remaining of the trial "A" and "B" sera were also included. The samples of rooster plasma were pooled in a similar manner.

Undiluted antiserum was mixed with an equal volume of the undiluted homotypic vaccine. Similar mixtures were made of undiluted antiserum with 1/10 or 1/100 dilutions of vaccine, and of undiluted vaccine with 1/3 or 1/10 dilutions of antiserum. All serum–virus mixtures were incubated for 2 hours at 37° C after which 0.2-ml amounts of the mixtures were added per tube culture of monkey kidney cells containing 1.8 ml of fresh medium. The inoculated cultures were observed microscopically and held for 14 days. At 7 days the medium on each culture was replaced by 2 ml of fresh medium containing the same amount of antiserum present initially. The accumulated results of several such tests are listed in Table IV.

TABLE IV

Neutralization of attenuated polioviruses by rabbit and rooster antisera

| Virus type | $Log_{10}$ pfu virus* per 0.1 ml | Dilution of antiserum | Tube cultures† protected 14 days by antisera | |
|---|---|---|---|---|
| | | | Rabbit | Rooster |
| I | 5.1 | Undiluted | 36/36 | 24/24 |
| | 6.1 | " | 36/36 | 24/24 |
| | 7.1 | " | 36/36 | 24/24 |
| | 7.1 | 1/3 | 36/36 | 24/24 |
| | 7.1 | 1/10 | 36/36 | 22/24 |
| II | 4.9 | Undiluted | 26/26 | 24/24 |
| | 5.9 | " | 26/26 | 24/24 |
| | 6.9 | " | 26/26 | 24/24 |
| | 6.9 | 1/3 | 26/26 | 24/24 |
| | 6.9 | 1/10 | 11/26 | 8/24 |
| III | 5.1 | Undiluted | 26/26 | 24/24 |
| | 6.1 | " | 26/26 | 24/24 |
| | 7.1 | " | 26/26 | 24/24 |
| | 7.1 | 1/3 | 13/26 | 24/24 |
| | 7.1 | 1/10 | 0/26 | 8/24 |

*Equal volumes of virus and antiserum were mixed and incubated for 2 hours at 37° C.
†Each tube culture of monkey kidney cells was inoculated with 0.2 ml of serum–virus mixture.

## Discussion

Although it has been stated that these attenuated strains of polioviruses grow poorly in human cells (6), it was found that, under the conditions described above, titers of $10^{7.5}$ pfu per ml were readily obtained (Table I). The original seed vaccines which had been grown in monkey kidney cells were assayed by the same method and found to contain $10^{7.5}$, $10^{7.6}$, and $10^{7.1}$ pfu per ml for types I, II, and III, respectively.

When antigens which had been grown in human kidney cells were administered intravenously to rabbits (Table II) in four doses of 2 ml, the resulting antibody titers 1 week after the fourth dose ("B" sera) averaged from 3000 to 11,000 depending on the type. With the same schedule of immunization but using one-half the amounts of antigen, even higher titers of 10,000 to 22,000 were reached in roosters (Table III) by 7 weeks. A further booster dose, given 1 month later, generally failed to improve the titers reached in rabbits or roosters. Roosters responded particularly well to the type III antigen.

Antisera produced by the above schedule of immunization were of sufficiently high titer to neutralize equal volumes of undiluted vaccine containing at least $10^8$ pfu per ml (Table IV). In fact, it was found that five of the six pooled antisera could be diluted threefold before mixing with the vaccines. Such mixtures may be used for the safety testing of oral vaccines in monkey kidney cultures.

## Acknowledgment

The authors wish to thank Miss C. R. Bowes and Mr. R. E. Armstrong for valuable technical assistance.

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# A RABIES VACCINE FROM HAMSTER KIDNEY TISSUE CULTURES: PREPARATION AND EVALUATION IN ANIMALS[1]

PAUL FENJE

## Abstract

A strain of fixed rabies virus adapted to hamster kidney tissue cells has produced culture fluids of high infectivity for rabbits and mice. These culture fluids were rendered non-infective by treatment with formaldehyde at a concentration of 8 m*M*. Rabbits immunized with this material produced antirabies antibody to a high titer and were subsequently proved to be resistant to intramuscular inoculation of rabies virus from the salivary glands of a naturally infected fox.

## Introduction

The ideal method of preventing rabies both in man and animals is to induce active immunity by means of a living strain of virus which has become permanently attenuated without loss of its immunogenic properties. For veterinary purposes, the chick-embryo-adapted Flury strain approximates to this ideal, and extensive experience under field conditions has shown it to be both effective and innocuous. For human prophylaxis, however, this type of vaccine has not advanced beyond the research stage. At present, therefore, we must still rely upon a vaccine which has been inactivated to the extent that it will pass the prescribed tests for absence of infectivity. Two rabies vaccines which fall into this category are the duck embryo vaccine developed by Peck, Powell, and Culbertson (8) and the type exemplified by the Semple vaccine, which is a phenolized suspension of infected rabbit brain. It is not possible to come to any definite conclusion about the efficacy of the classical Pasteur treatment or of the Semple vaccine. On the evidence provided by laboratory tests employing immunization followed by challenge, there can be no doubt about the value of these vaccines; but experiments of this kind are artificial and do not reflect the conditions resulting from the bite of a rabid animal.

A serious disadvantage of most antirabies vaccines is the fact that they contain large amounts of host protein in proportion to their content of virus protein. In the case of the Semple vaccine, for example, the daily dose of the Connaught Medical Research Laboratories rabies vaccine is 2 ml of a 4% rabbit brain suspension. This represents 0.08 g of brain tissue. The amount of virus protein contained in this quantity of tissue cannot be determined exactly but it must be extremely small in view of the infectivity titers as determined by experiment. The usual infectivity figures for a fixed virus are $10^7$ to $10^8$ m.i.d. per gram of brain substance as determined by intracerebral inoculation of white mice. The antigenic stimulus provided by this quantity of virus must be very feeble in comparison with that provided by the associated 0.08 g of host protein. A similar objection, though less serious, can be made against vaccines prepared from avian embryo tissues. Glenny and his co-workers

[1]Manuscript received July 22, 1960.
Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

(3, 4, 5) showed many years ago that a crowding-out effect becomes apparent when multiple antigens are injected, if one or more is present in excessive concentration; the antibodies produced are limited to those antigens that were employed in greatest amounts. It is reasonable to suggest that this crowding-out effect may be an important factor in determining the response to a rabies vaccine.

These considerations lead to the conclusion that if an inactivated rabies vaccine must be employed for human prophylaxis the amount of host cell protein should be substantially reduced in proportion to the amount of virus protein. A fairly simple method of achieving this is to propagate the virus in tissue cultures of hamster kidney cells and to use the culture fluids as starting material for the vaccine. Some experiments on these lines are here recorded.

## Experimental

The methods used in this laboratory to propagate rabies virus in hamster kidney tissue cultures have recently been described in a separate communication (1). The following is a summary of the technique.

Young healthy hamsters about 4 weeks of age are sacrificed and the kidneys are removed for immediate processing. The kidney tissue is first cut into fragments and then subjected to mild tryptic digestion. Usually, 30–60 minutes at 37° C in 0.25% trypsin at pH 7.2 is sufficient to produce a well-dispersed cell suspension. After thorough washing in phosphate buffer – saline followed by a second washing in culture medium, the cells are distributed into Pyrex test tubes (160 mm × 28 mm) closed with a tightly fitting silicone stopper. The medium consists of Hanks' balanced salt solution with lactalbumin hydrolyzate 0.5% and horse serum 15.0%. Each culture tube receives 10.0 ml of this medium together with cell suspension equivalent to 250,000 cells. The tube is then incubated at 37° C in a position slightly inclined to the horizontal. When a cell monolayer has formed the medium is changed, the infecting dose of virus is added, and a dialysis assembly (1) is fitted into the culture tube in place of the silicone stopper. The dialysis tube is then filled with 25 ml of culture medium and closed with another stopper. The whole apparatus is now ready to be returned to the incubator. At intervals of 4 to 6 days the fluid in the dialysis tube is removed and replaced by fresh medium: the cell monolayer is not disturbed. In this apparatus it is possible to maintain the cells in a healthy condition for periods up to 10 weeks.

The virus strain SAD was again used in these experiments. The origin of this strain and also the method used to adapt it to hamster kidney tissue cultures have been described in the previous communication (1). Three to four weeks after infection of the kidney cells the culture fluids were harvested, pooled together, and then lightly centrifuged to remove cell debris. A sample of supernate was immediately assayed by intracerebral injection of mice. Formaldehyde solution (analytical reagent, 37% HCHO) was then added to the remainder to bring the concentration of HCHO to 0.008 *M*. At the same time, glycine was added to a concentration of 0.02 *M*: this is believed to act as

a stabilizer, maintaining the concentration of free HCHO at an approximately constant level (Neumuller (7), Gard (2)). In practice, it was convenient to add 1.0 volume of 3.38% formaldehyde solution containing 1.0 *M* glycine to 49 volumes of the infected tissue culture fluid. After the solution had been allowed to stand for 6 hours at 37° C the residual HCHO was removed by 2 stoichiometric equivalents of $NaHSO_3$. This fluid, after the addition of merthiolate to a concentration of 1 in 10,000, constituted the finished vaccine. It was not used for immunization if the infectivity prior to the addition of HCHO was less than $10^6$ $LD_{50}$ doses/ml as determined by intracerebral inoculation of white mice. Each batch of vaccine was tested for absence of infectivity by injecting a sample intracerebrally into rabbits and mice; all samples proved to be innocuous.

*Immunogenic Potency Tests*

For the vaccination trials young adult rabbits, weight 4–5 lb, were used. The full course of immunization consisted of 10 subcutaneous injections each of 1.0 ml of formalinized vaccine given on 10 consecutive days without adjuvant. Blood samples were taken before immunization and again on the day following the last injection of vaccine. A third blood sample was obtained 2 weeks later. The immunity invoked by this series of injections of vaccine was challenged by inoculating a strain of rabies virus obtained from the salivary gland of a naturally infected fox. This test was chosen in preference to one of the better known methods because it is less stringent than one which employs the intracerebral route and also because it has given satisfactory results in the evaluation of rabies vaccines prepared for veterinary purposes. The dose was 0.3 ml of a 1 in 10 suspension of finely minced gland, injected into the masseter muscle of the immunized animal on the day following the last injection of vaccine. This challenge dose of virus represented about $2 \times 10^4$ m.i.d. as determined by intramuscular inoculation of guinea pigs, or $10^8$ $LD_{50}$ intracerebral doses for mice.

The blood samples obtained from the immunized rabbits were assayed for antibody content by means of a virus–serum neutralization test. The details will be given in the next section.

## Results

Four batches of formalinized vaccine having the required original infective potency of at least $10^{6.5}$ $LD_{50}$ doses/ml were prepared. Separate immunization experiments were carried out on each batch, but as the methods of preparing and evaluating these vaccines were exactly the same in each case it will be convenient to present the results as a single composite experiment. The details are as follows.

A total of 25 rabbits received the full course of 10 daily injections of 1.0 ml of vaccine. These 25 immunized animals together with 18 normal rabbits as controls then received the challenge dose of fox salivary gland rabies virus. All 25 vaccinated animals resisted the challenge and remained normal throughout an observation period of 6 to 8 weeks. All 18 control animals developed

signs characteristic of rabies: paralysis commenced in the neck muscles and fore limbs about the 12th day and death occurred on the 15th to the 20th day with complete paralysis. The blood sera taken from the 25 immunized rabbits were submitted to serum–virus neutralization tests. A preliminary screening test was first performed, using 100 $LD_{50}$ doses of virus (strain CVS) and 0.015 ml of serum, these being mixed and inoculated intracerebrally into mice in a volume of 0.03 ml 1 hour later. The preliminary test showed that neutralizing antibody was present in the postimmunization samples of every one of the 25 animals. The sera were then submitted to a quantitative virus neutralization test. The same test dose of virus was contained in final serum dilutions of 1 in 5, 1 in 25, and 1 in 125; and after incubation for 1 hour at 37° C, 0.03 ml quantities were injected intracerebrally into mice. The animals thus received 100 $LD_{50}$ doses of virus together with 0.006 ml, 0.0012 ml, or 0.00024 ml of the serum to be tested. Each serum–virus mixture was inoculated into four animals. The results of these tests demonstrated that appreciable amounts of antibody were present in these sera. A summary of these results is given in Table I. It will be noted that there was little difference in neutralizing ability between the first and second postimmunization blood samples.

TABLE I

Response of immunized rabbits to challenge with fox rabies virus and antibody content of serum samples taken from these animals

| Batch number of tissue-culture vaccine | Number of vaccinated rabbits | Number of normal rabbits (controls) | Result of challenge | Serum–virus neutralization tests (mouse intracerebral test). Reciprocals of serum dilution of which 0.03 ml protected against 100 $LD_{50}$ units of rabies strain CVS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Samples from individual rabbits taken after completion of immunization | | | | Samples from individual rabbits taken 2 weeks later | | | |
| No. 54 | 4 | 4 | S | >125 | 125 | 17 | 11 | 125 | 25 | 8 | 56 |
| | | | D | | | | | | | | |
| No. 58 | 4 | 4 | S | 42 | 42 | 36 | 14 | 44 | 25 | 8 | 42 |
| | | | D | | | | | | | | |
| No. 59 | 3 | 5 | S | 42 | 125 | >125 | | 56 | >125 | >125 | |
| | | | D | | | | | | | | |
| No. 62 | 14 | 5 | S | 42 | >125 | >125 | | 14 | >125 | >125 | |
| | | | D | | | | | | | | |

NOTE: S = survived, D = died.
The challenge dose (injected into the masseter muscle) was the equivalent of $2 \times 10^4$ m.l.d. as determined by intramuscular injection of guinea pigs. The serum titers are 50% end points of rabies mortality as determined by the method of Reed and Muench. In the case of vaccine No. 62 the sera of only 3 of the 14 immunized rabbits were titrated.

As an additional control to the experiment described above, two of the non-immune rabbits were bled 2 weeks after inoculation with the challenge virus immediately before the onset of paralysis. There was no detectable antibody in the sera at that time. This was accepted as evidence that the virus injected as the challenge was not responsible for the immune body found in the sera of animals which had received the formalinized vaccine.

## Comment

These results are encouraging but further work must be undertaken before the value of this vaccine can be assessed, particularly with respect to the measurement of the immune response. Although it has not been employed

widely, the use of rabid fox salivary gland for challenge tests has much to recommend it. This method is probably the nearest possible approximation to the natural mode of infection, and it has the added advantage that the infecting dose used as challenge can be determined accurately. In further experiments now in progress we are comparing this method with some of the tests recommended by the Expert Committee on Rabies, World Health Organization (6).

Hamster kidney tissue culture vaccine is a water-clear fluid. The only constituent likely to cause a reaction on injection is the horse serum which is an essential part of the culture medium. The presence of serum in the vaccine would make it unsuitable for injection into man because natural sensitivity to horse protein is not uncommon; and a 10- to 14-day course of injections, if this were the method of immunization recommended, would certainly produce sensitization in some of those undergoing treatment. It will therefore be necessary to modify the present culture methods in a manner which will allow the virus to propagate in hamster kidney cells growing in a fully synthetic medium.

The daily injection schedule, now almost universally employed for immunization against rabies, is dictated by the urgent necessity of producing immunity as rapidly as possible. A potent vaccine almost free from host protein may make it possible to use a more convenient schedule without sacrifice of effectiveness. A large amount of the antigen incorporated in a depot substance may be the means of achieving this. Finally, it seems reasonable to believe that the new vaccine, being devoid of nerve tissue, can be given without fear of a neuroparalytic reaction.

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Dr. J. F. Crawley and his staff of the Veterinary Division of these Laboratories supplied the fox rabies virus and provided information about the infectivity of this material. This help is gratefully acknowledged.

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# GROWTH RESPONSE OF CHLAMYDOMONAS AND HAEMATOCOCCUS TO THE VOLATILE FATTY ACIDS[1]

RAYMOND G. STROSS[2]

## Abstract

The first five straight-chain saturated fatty acids were tested to determine their effect on cell division of phytoflagellates. *Chlamydomonas reinhardti* Danseard and *Haematococcus pluvialis* Flotow were selected for measurement because of information available from similar studies of the long-chain acids. Cultures were grown at five concentrations of the sodium salt within the range of 0.5 to 10.0 mmoles/liter.

Acetate stimulated the rate of cell division, while butyrate, the other acid with an even number of carbons, produced an inconsistent although negligible effect.

The fatty acids with an odd number of carbons inhibited division of cells, with propionate being most and valerate least effective. Inhibition in these tests, in which initial pH was kept constant, was proportional to the initial concentration of the acid salt. Additional tests showed propionate inhibition to be approximately proportional to a calculated amount of undissociated acid in the medium. *H. pluvialis* was 9–12 times more sensitive than was *C. reinhardti*.

The possibility of propionic acid inhibition of algae in acid lakes is discussed.

## Introduction

Lipids produced by algae in culture are thought to be autoinhibitory to division of the cells (14, 15, 16). The partially oxidized products of long-chain unsaturated fatty acids are thought to be responsible (20), although the saturated acids are also effective inhibitors of growth (17). Proctor (17) concluded that higher fatty acids may be responsible for previously observed instances of inhibition in algal cultures. One necessary condition, however, appears to be a strongly alkaline medium, which probably is necessary for liberation into the medium of sufficient amounts of fatty acids from cell glycerides. This requirement of high pH may impose a severe restriction on the effectiveness of long-chain fatty acids in most unpolluted lakes.

That volatile fatty acids inhibit growth of algae and protozoa has been known for a long time and is discussed in older reviews of the subject (3, 10). These acids appear to be less toxic than those of longer chain-length, although their toxicity is known to increase at low pH of the medium. The so-called acetate flagellates appear to be especially resistant even in acid media (9). Growth of algae in axenic culture probably is not inhibited by the volatile fatty acids because little if any is produced (1, 12). On the other hand, algae in lakes are exposed to these acids and may be influenced by the presumably small concentrations encountered there. Two species of flagellates, one of known resistance, the other quite sensitive, were employed in this study to demonstrate the differential influence of the volatile fatty acids on the growth of algae and the importance of the acidity of the medium in controlling degree of response.

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## Methods

Pure cultures of *Chlamydomonas reinhardti* Danseard I. U. strain No. 89 and *Haematococcus pluvialis* Flotow, obtained from R. R. L. Guillard, were used. The normal acids, formic through valeric, added as the sodium salt, were tested in a range of concentration of 0.5 to 10.0 mmoles/liter. The culture medium for tests which compared the effect of the five acids was adjusted to pH 7.25 initially, but it became progressively acid with growth of the culture. To determine effect of the acids, the division rate of treated and control cultures was compared at the end of log growth of the control cultures.

In evaluating the influence of pH of the medium on the degree to which propionate retarded division rate, the cultures were grown for a 30-hour period during which the populations had grown sufficiently for accurate measurement, but the uptake of salts from the medium had not been sufficiently great for a change in pH to occur. Tris buffer was ineffective in a part of the range of pH employed in these experiments, but was added for the sake of uniformity.

Both species were grown in the same inorganic medium (Table I). All cultures were suspended in 50 ml of medium contained in 125-ml cotton-stoppered flasks. They were placed in 250–300 foot-candles of continuous light which was provided by fluorescent lamps of daylight type. In the tests comparing the effect of the various acids, *Chlamydomonas* was grown at 18° C and *Haematococcus*, at 23° C. The latter temperature was used in testing the response of *Chlamydomonas* to the fatty acids at different pH.

TABLE I

Culture medium

| | |
|---|---|
| $NH_4NO_3$ | 300 mg |
| $K_2HPO_4$ | 100 |
| $MgSO_4$ | 200 |
| $CaCl_2$ | 100 |
| Fe sequestrene (13% Fe) | 4.0 |
| Tris buffer | 400 |
| Trace elements | 1.0 ml |
| Double distilled $H_2O$ | 1000 |

NOTE: Cultures were adjusted to pH desired with dilute hydrochloric acid.

Division rates were determined by cell counts at inoculation and at harvest (4.0 days). Four replicate counts of each duplicate (eight samples) were made in a Levy haemocytometer (11). Results are expressed as a percentage of the number of divisions per day in the control cultures.

## Results

With the exception of butyrate, the response of *Chlamydomonas* in replicate assays was uniform. Propionate, formate, and valerate retarded growth in proportion to the initial concentration of acid salt, with the degree of inhibition being in the order mentioned (Table II).

TABLE II

Effect of straight-chain volatile fatty acids on division rate of *Chlamydomonas reinhardti* at initial pH 7.25. Values are listed as percentage of division rate of control cultures at the end of log growth (ca. 1.35/day)

| | Millimoles/liter of acid salt | | | | |
|---|---|---|---|---|---|
| | 0.5 | 1.0 | 2.0 | 4.0 | 10.0 |
| Propionate | 96 | 88 | 81 | 67 | 35 |
| Formate | 98 | 95 | 92 | 88 | 72 |
| Valerate | 102 | 100 | 99 | 98 | 97 |
| Butyrate | 100 | 101 | 100 | 100 | 97 |
| Acetate | 109 | 111 | 111 | 112 | 114 |

Acetate stimulated growth at all concentrations, the smallest level used resulting in a marked stimulation. Because of an increase in pH of the cultures with growth of the cells, a phenomenon peculiar to acetate, the larger amounts of acetate were not effective in proportion to the initial concentration, probably because of pH inhibition. The results from the tests of butyrate, although unsatisfactory, suggested that this acid present in the range of concentration employed in this study has negligible effect on the division rate of *Chlamydomonas*. In these tests the division rate of *Chlamydomonas* in the controls was 1.35 times per day, being slightly more or less depending on time of harvest. The fact that acetate stimulated the rate of cell division indicated a faster rate is possible at 18° C with the provision of higher intensities of light or an enrichment of $CO_2$.

*Influence of pH*

In this, as in other studies (7, 8), pH of the medium controlled the degree of inhibition by the fatty acid. Erickson *et al.* (7) showed the response of *Chlorella* to acetic acid to be nearly proportional to the concentration of undissociated acid when pH was equal or greater than the p$K$ of the acid, a response demonstrated for many kinds of weakly dissociating organic acids (18). Because propionate provided the severest inhibition in this study, it was used to test the degree of inhibition at several concentrations of the free acid. These were provided by adjustment of initial pH at three to five levels, depending on the test, within the range of pH 6.60 and 7.25. In calculating the concentration of free acid, the nutrient salts which were present in the medium were assumed not to interfere with the proportion of dissociated and undissociated acid as expressed in the Henderson-Hasselbalch equation. In the 30-hour tests 2.0 mmoles/liter of propionate were added.

The inhibition caused by propionic acid was roughly proportional to its concentration in the medium (Fig. 1). The degree to which cell division of *Chlamydomonas* was inhibited was, however, dependent on the age of the cells used for inoculation. Cells from stationary-phase inocula were less sensitive than were those from log-phase. Moreover, resistance, or lack of depression in division rate, appeared to increase with the length of the period in which inoculum cultures were in stationary phase. Resistance to the free acid may be explained as a result of the cells dividing, owing to a reserve of

food stored in the older cells. Because the cultures divided approximately two times, depending on age of inocula, during the 30-hour "incubation" period, a reserve of food may shorten the time required for the initial division.

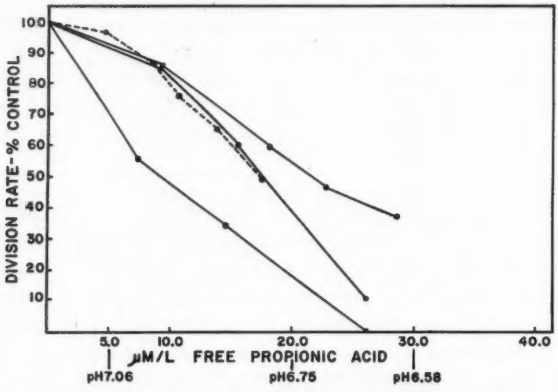


FIG. 1. Inhibition of division rate of *Chlamydomonas* cells grown in the presence of propionic acid expressed as percentage of rate in the control. Solid lines are for cultures containing 2.0 mmoles/liter of the acid salt. The dotted line represents additions of 1.0 mmole/liter. From left to right the lines indicate results of testing 3-, 5-, and 8-day-old inocula (dotted line results from 5-day inoculum). (Note that pH values apply only to solid lines or to an acid-salt concentration of 2.0 mmoles/liter.)

Similar tests run on *Haematococcus* showed it to be more sensitive to propionic acid. Concentrations of 0.05, 0.1, 0.2, and 0.3 mmoles/liter of the acid salt were tested in a range of pH similar to that used for *Chlamydomonas*. The procedure differed in that the cultures were permitted to incubate until the controls reached the end of log-phase growth. The use of initial pH for calculating the amount of undissociated fatty acid in the medium was considered permissible since growth and, therefore, change in pH were small in cultures where the fatty acid was effective. Division rate in the controls was 1.2 times per day.

Growth of *Haematococcus* in the presence of propionic acid is expressed as a function of pH and of the calculated amount of free acid (Fig. 2). The sensitivity of this species may be illustrated by comparison of rate of cell division in 0.1 and 0.2 mmole/liter of the acid salt at pH 6.6. At the former concentration cell division was scarcely affected, whereas at 0.2 mmole/liter inhibition was nearly complete. This species was approximately 10 times more sensitive to free propionic acid than was *Chlamydomonas*. The much greater sensitivity of *Haematococcus* to the volatile fatty acids, which was shown to exist for pelargonic and capric salts (17), was not true in tests of longer-chain fatty acids in which case the sensitivity of the two species to the presence of the salts was rather comparable (17).

In conjunction with the above experiments the medium from senescent cultures of *Chlorella* was examined for total concentration of fatty acids.

*C. pyrenoidosa* I. U. No. 251 and *C. vulgaris* (a strain isolated by J. McLachlan) were grown in a medium and atmosphere which produce a high content of fats in the cells (12, 19). After the cultures had remained for 1 month in a senescent state, which was indicated by the fading of chlorophyll in *C. pyrenoidosa*, the cultures were harvested by centrifugation, and the fatty acids in the cell-free medium prepared for measurement by titration in a standard manner (2). The density of cells at this time was in the range of 70–90 million cells/ml. At pH 5.0–6.0, the range in which the cells were grown, the average content of fatty acids in the medium of five cultures was 4.5 μeq/liter with a range from 3.0 to 6.9 μeq/liter. Because there seemed to be no difference in the amount existing in the medium of the two species of *Chlorella*, the average may be representative for cultures at similar pH, cell density, and nutrient medium.

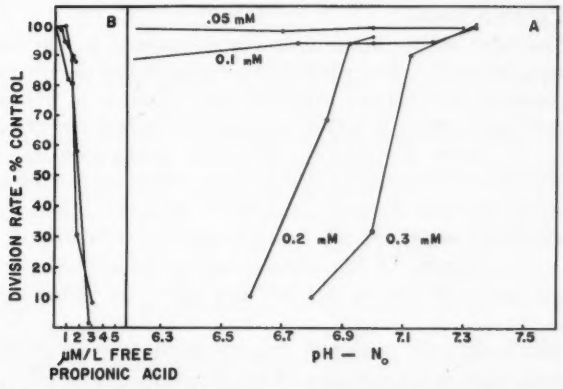


FIG. 2. Inhibition of division rate of *Haematococcus* cells grown in the presence of propionic acid. Portion A of the graph shows inhibition of acid salt as a function of pH at inoculation. Response at the four concentrations of acid salt is expressed as percentage of division rate in control cultures. Portion B shows response as a function of the calculated amount of free propionic acid in the medium. (Note: These tests differ from those in Fig. 1 in that the cultures were incubated until the stationary phase of growth was reached in the controls. Considered at the end of log growth, a division rate of 80% that of the control is roughly equivalent to an LG-50.)

On the basis of Milner's analysis (12) of the fatty acid composition of *C. pyrenoidosa* only a trace of the total acids liberated was volatile. Assuming an average molecular weight of palmitic acid, a value consistent with the findings of Milner, the medium contained approximately 1.2 mg/liter of total fatty acid. This would seem to be an inadequate amount to cause inhibition of cell division of *Haematococcus* even if the entire 1.2 mg were present as propionic acid. Whether the higher fatty acids in the *Chlorella* medium would affect cell division of *Haematococcus* at strongly alkaline pH would depend on the proportion of saturated acids which are considered to be the more toxic (4). It should be pointed out that larger quantities of fatty acids may accumulate in the medium of *Chlorella* cultures grown at alkaline pH (17).

## Discussion and Conclusions

The response of *Chlamydomonas* and *Haematococcus* to dilute concentrations of the completely water-soluble fatty acids is correlated with the number of carbon atoms in the molecule, a fact which has been demonstrated on other flagellates (5). Formic and propionic acids, with an uneven number of carbon atoms, inhibit growth, whereas acetate is stimulatory at least under culture conditions used in this study. Because the degree of inhibition is proportional to the amount of free acid in the medium, an assay of formate at the same pH as propionate would not be expected to show an equal response, owing to the difference in p$K$ of the two acids. At the pH of these assays there was supposedly 10 times more propionic acid in the medium. A possibly greater permeability of the cell to formic acid, however, does not permit a rigorous comparison to determine the actual effect of the two fatty acids. Butyrate and valerate, because of their limited solubility in water, are not considered in the discussion.

An active uptake of the acetate ion is suggested in the assays with *Chlamydomonas*. Normally, the cells prefer ammonium to the nitrate ion when the two are present together, as indicated by a decline in pH with growth of the culture. When acetate is added, pH of the medium increases markedly. Since there is no reason to suspect a change in preference, the increase in pH suggests the uptake of acetate is greater than the uptake of ammonium ion. If passage of acetate were by simple diffusion, then the pH in cultures with the other completely water-soluble acids should have behaved in a similar manner. An active uptake of the acetate ion would explain the increasing effect of the dissociated acid at pH 5.0 and above as noted for *Chlorella* elsewhere (6).

Although propionic acid probably is never inhibitory to growth of algae in axenic culture, there is a possibility of it having an adverse effect on natural populations of algae under certain circumstances. Lakes and ponds which receive drainage from the manures of ruminants should contain propionic acid because its production in the rumina of cattle is second only to acetic (21). Although propionic acid is not reported in the older analyses of unpolluted lakes (22), more recent work demonstrates trace amounts in river waters (13). An appreciable production may be expected in lakes in which water near the bottom is devoid of oxygen for considerable periods each year and, thus, favorable for microbial excretion of the volatile acids. The most effective situation may be in bog lakes which combine anaerobiosis with low pH. In any event the effect, because of the presumably small concentration of the acid, is likely to be one of providing the more acid resistant species with a competitive advantage owing to the limited rate at which crucial nutrients are supplied.

## Acknowledgments

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# THE EFFECT OF CARBAMYL PHOSPHATE AND CYANATE ON ESCHERICHIA COLI[1]

ANDREW TAUSSIG[2]


## Abstract

Experiments have shown that 0.02 *M* synthetic carbamyl phosphate inhibits the growth of *Escherichia coli* when added in the lag phase but not when added in the logarithmic growth phase. Conversely, potassium cyanate at similar concentrations was found to cause only an elongation of the lag phase but when added during the logarithmic growth phase it was found to inhibit further growth of the cells. Both inhibitions were found reversible by addition of glutathione and cysteine. Carbamyl cysteine formation is offered as the possible explanation for the inhibitory property of carbamyl phosphate since the inhibition caused by carbamyl cysteine was also found to be reversible by additions of SH compounds and since carbamyl phosphate plus cysteine was found to produce carbamyl cysteine.


## Introduction

It was previously reported that *Escherichia coli* grown anaerobically does not support the growth of the phages $T_1$ and $T_2$ (11). The addition of glucose plus pyruvate or oxaloacetate to the broth growth medium resulted in phage development upon infection of the anaerobically grown host cells. During attempts to bring about phage synthesis in anaerobically grown *E. coli* by the addition, to the growth medium, of substances which possess a high energy phosphate bond, carbamyl phosphate was tested. However, it was found that addition of carbamyl phosphate to the growth medium resulted in the inhibition of growth of *E. coli*, not only under anaerobic but also under aerobic conditions.

An investigation has been undertaken to explain this phenomenon, and the present paper reports the result of this investigation.

## Materials and Methods

### *Bacteria*

*Escherichia coli* strain B/1,5 was derived from strain B of this organism, which was obtained from the late Dr. M. Adams of New York University. The Duncan strain of *Micrococcus pyogenes* was received from Dr. E. H. Creaser of this laboratory. The bacteria were maintained on nutrient agar slopes and subcultured daily before use.

### *Media*

Difco dehydrated nutrient broth, reinforced with 2 mg per ml of glucose, was used for growing the cells in most experiments. This medium will be referred to as nutrient broth – glucose. The synthetic medium M9 was prepared as described by Herriott and Barlow (4).

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Contribution from the McGill – Montreal General Hospital Research Institute, 3619 University Street, Montreal 2, Que.
[2]Fellow of the Canadian Life Insurance Officers Association.

*Chemicals*

Lithium carbamyl phosphate was prepared according to the method of Jones *et al.* (5). The sodium salt of this compound was made by converting the lithium salt on a Dowex 50 column. Orthonitrophenyl β-D-galactoside (ONPG) was obtained from the California Corporation for Biochemical Research. All other chemicals were reagent grade and were obtained commercially.

*Methods*

Bacterial growth was determined turbidimetrically.

Carbamyl phosphate (CP) was determined as described by Jones *et al.* (5) on the basis of hydrolyzable phosphate and also on the basis of its ability to form citrulline from ornithine in the presence of *E. coli* extracts. The extracts were prepared by breaking up *E. coli* cells with glass beads in the shaker head of the International Refrigerated Centrifuge.

The formation of β-galactoside was determined according to the method of Lederberg (6).

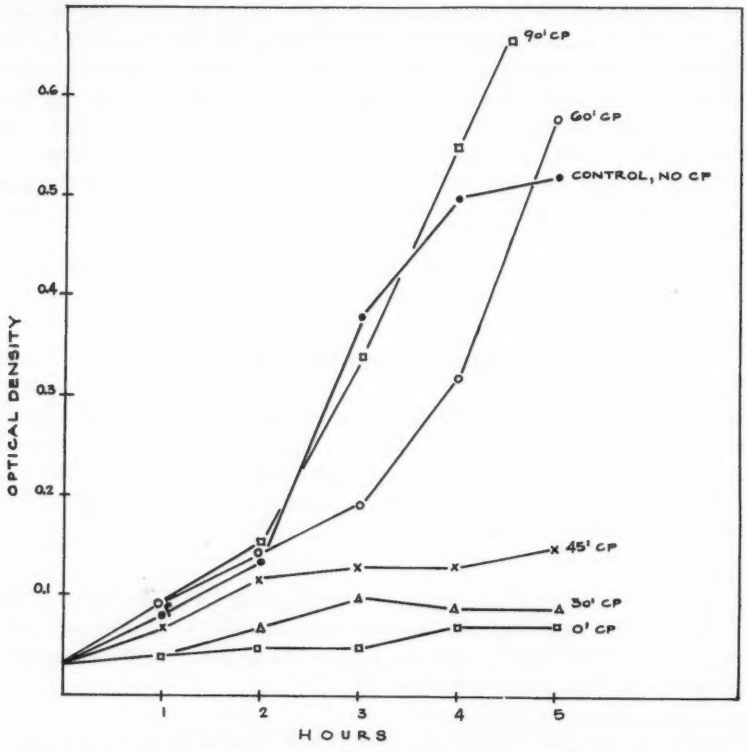


FIG. 1. The effect of the time of addition of 0.02 *M* carbamyl phosphate (CP) to nutrient broth on the growth of *E. coli* B/1,5; 0.02 *M* carbamyl phosphate was added at the indicated times after inoculation with about $10^6$ cells/ml.

## Results

*Effect of Carbamyl Phosphate on the Growth of E. coli*

*E. coli* B/1,5 was grown in nutrient broth – glucose. Carbamyl phosphate at a concentration of 0.02 *M* was added to the growth medium at different times during the growth cycle. It can be seen from the results shown in Fig. 1 that 0.02 *M* carbamyl phosphate inhibits the growth of the cells if it is added to the broth during the first 45 minutes after inoculation. Addition of 0.01 *M* carbamyl phosphate to the growth medium had an effect similar to 0.02 *M* carbamyl phosphate, but 0.005 *M* carbamyl phosphate permitted nearly normal growth.

*Possible Mode of Action of Carbamyl Phosphate*

The carbamyl phosphate used in these experiments was the synthetic lithium salt. It was possible that the inhibition was due to the lithium ions. Lithium chloride therefore was tested for its action on bacterial growth. However, LiCl at a concentration of 0.04 *M* had no effect. The sodium salt of carbamyl phosphate was found to behave exactly like the lithium salt, so that it was concluded that the lithium ion is not responsible for the inhibition, and that the carbamyl moiety inhibited the growth.

It was found by Fitzgerald (3) that 10 mg% cyanate inhibited the growth of *Mycobacterium tuberculosis* in Dubos medium. Carbamyl phosphate under

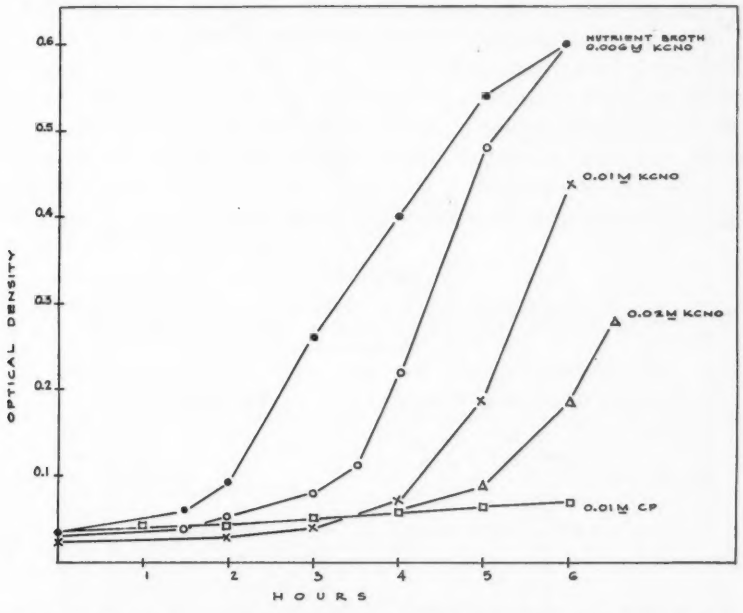


FIG. 2. Effect of increasing concentrations of KCNO on the growth of *E. coli* B/1,5; cyanate at the indicated concentrations was added at the time of inoculation with about $10^6$ cells/ml.

certain conditions breaks down to cyanate plus inorganic phosphate (9). It was thus possible that the inhibition is attributable to the cyanate ion. The effect of KCNO was therefore tested on the growth of *E. coli*.

The effects of addition of increasing concentrations of KCNO to the growth medium are shown in Fig. 2. It can be seen that cyanate behaves differently than carbamyl phosphate, for the bacteria soon overcome cyanate inhibition and start to grow after 3 or 4 hours, whereas, when carbamyl phosphate is present in the medium at the time of inoculation, growth is inhibited throughout the experimental periods used, as illustrated by the 5-hour '0 time' curve in Fig. 1, and particularly by the CP curve in the longer 6-hour experiment shown in Fig. 2. Further, as may be seen in Fig. 3, the addition of cyanate to logarithmically growing *E. coli* stops the growth, while addition of carbamyl phosphate to the growth medium of *E. coli* during the logarithmic growth phase has no effect. However, it must be mentioned that addition of carbamyl phosphate to logarithmically growing *E. coli* cells in the synthetic medium M9

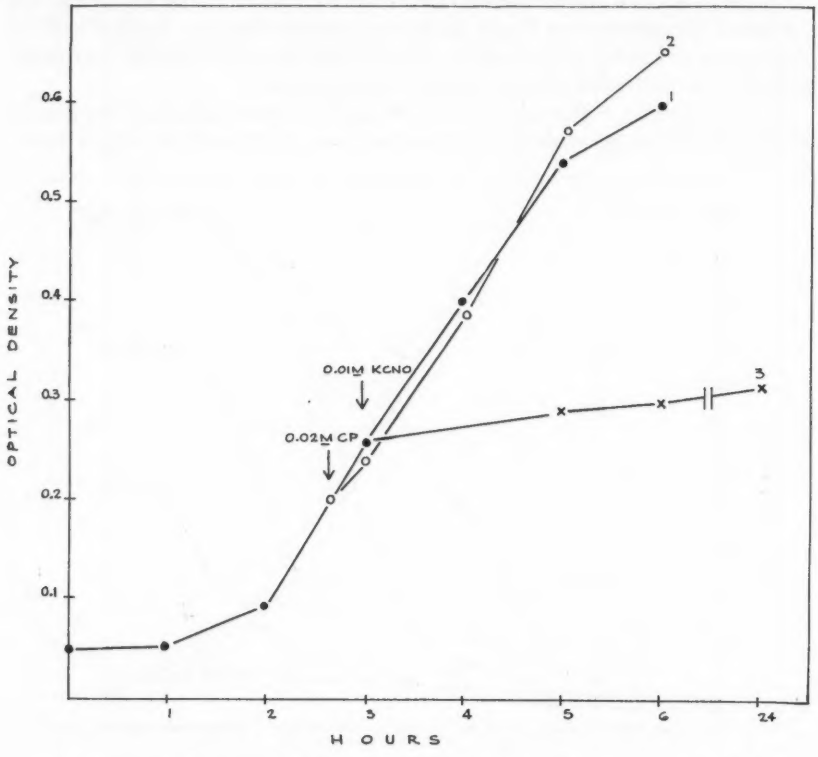


FIG. 3. Effects of addition of KCNO or carbamyl phosphate to bacteria in the logarithmic growth phase; *E. coli* B/1,5 was grown in nutrient broth and at the times indicated by the arrows 0.02 *M* carbamyl phosphate or 0.01 *M* KCNO was added to the growth medium. (1) No additions; (2) carbamyl phosphate; (3) KCNO.

inhibited the growth of the bacteria. This experiment was performed by adding glucose alone, or carbamyl phosphate together with glucose, to the growth medium at a time when growth had stopped due to exhaustion of glucose. Cell growth was resumed immediately in both cases, but it stopped within 10 minutes in the presence of 0.02 *M* carbamyl phosphate.

In order to investigate whether the actions of carbamyl phosphate in the lag phase and of KCNO in the logarithmic growth phase were bactericidal or bacteriostatic, viable cell counts were made. The number of viable cells remained constant in the presence of carbamyl phosphate. Additions of KCNO in the logarithmic growth phase permitted first a slight increase of about 25% in the number of cells in about 30 minutes. Subsequently, there was no further change for a period of up to 2 hours.

*Effect of Preincubation of Carbamyl Phosphate*

When carbamyl phosphate was preincubated with broth for 30 minutes prior to addition to the growth medium of logarithmically growing *E. coli*, there was no inhibition of growth although carbamyl phosphate activity was reduced by 65% as measured by citrulline formation, indicating that the carbamyl phosphate was hydrolyzed.

*Reversal of the Inhibition of Growth*

It was observed that when cyanate was added to bacteria growing in M9 plus caseine hydrolyzate, reinforced with tryptophane and cysteine, there was no inhibition of bacterial growth. This lack of inhibition was traced to the presence of the cysteine in the medium. Cysteine, when added to the broth, was also found to be capable of reversing the inhibition of growth of these organisms. On oxidation, cysteine precipitates, making turbidimetric measurements of growth difficult. Glutathione (GSH) was tried instead of cysteine with good results. Results shown in Table I demonstrate that even low concentrations of glutathione effectively reduce the increase in lag phase

TABLE I

Effect of glutathione on the length of the lag phase in the growth of *E. coli* B grown in nutrient broth in the presence of cyanate or carbamyl phosphate

| Additions | Length of lag phase (minutes) |
|---|---|
| None | 55 |
| 0.02 *M* CP | No growth |
| 0.02 *M* KCNO | 245 |
| 0.02 *M* CP + 0.02 *M* GSH | 75 |
| 0.02 *M* CP + 0.01 *M* GSH | 210 |
| 0.02 *M* CP + 0.005 *M* GSH | No growth |
| 0.02 *M* KCNO + 0.02 *M* GSH | 70 |
| 0.02 *M* KCNO + 0.01 *M* GSH | 90 |
| 0.02 *M* KCNO + 0.005 *M* GSH | 100 |
| 0.02 *M* KCNO + 0.0025 *M* GSH | 125 |
| 0.02 *M* KCNO + 0.00125 *M* GSH | 165 |
| 0.02 *M* KCNO + 0.000625 *M* GSH | 175 |

resulting from the addition of KCNO to the medium. Higher concentrations were necessary to effect the reversal of the total inhibition of bacterial growth brought about by carbamyl phosphate.

It was of interest to find out whether, to effect a reversal of the inhibition in bacterial growth, it is necessary to add glutathione simultaneously with the inhibiting compounds. Therefore, glutathione was added to the growth medium together with, and at various times after, the addition of KCNO or carbamyl phosphate. Figure 4 shows that there was an immediate reversal of the inhibitions caused by either KCNO or carbamyl phosphate when GSH

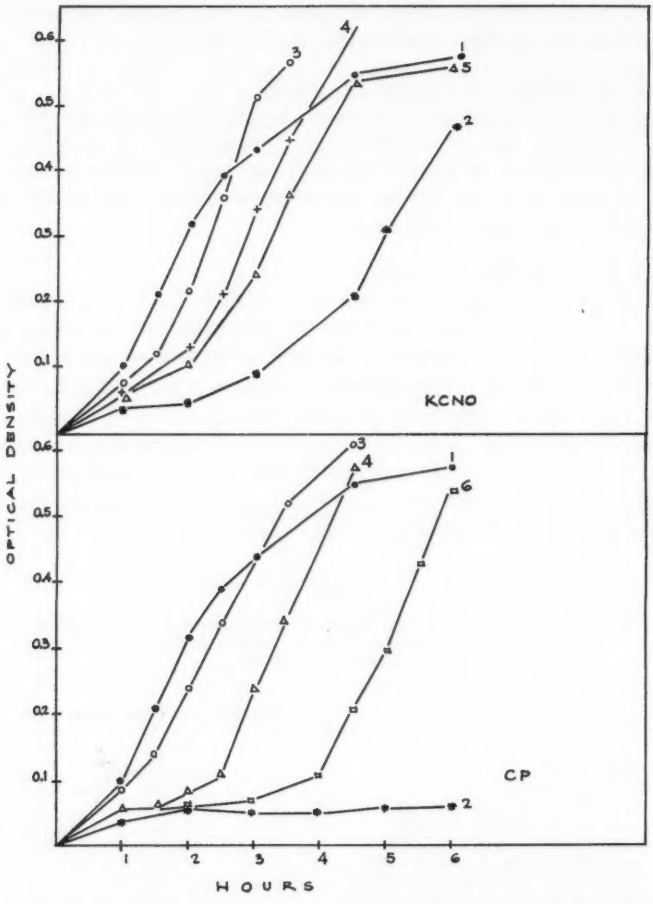


FIG. 4. The effect of the time of addition of 0.02 *M* glutathione on the growth of *E. coli* B/1,5, inhibited by 0.02 *M* KCNO or 0.02 *M* carbamyl phosphate in nutrient broth. (1) No additions; (2) carbamyl phosphate or KCNO alone; (3) GSH at 0 minutes; (4) GSH at 30 minutes; (5) GSH at 60 minutes; (6) GSH at 120 minutes.

was added simultaneously with the inhibitors, and that if the glutathione was added after an inhibition of growth was established, there was a lag phase of about 90 minutes in the case of carbamyl phosphate or one of 30 minutes in the case of KCNO before growth was resumed.

*Effect of Carbamyl-cysteine on Growth and Citrulline Synthesis in E. coli*

It has been reported by Ravel *et al.* (8) that S-carbamyl-L-cysteine is inhibitory to the growth of *Streptococcus lactis* and *Lactobacillus arabinosus*, and that it also inhibits the synthesis of citrulline by *S. lactis*. Results shown in Table II indicate that carbamyl cysteine inhibits the growth of *E. coli* B/1,5 as well and also that it inhibits citrulline synthesis in this organism. However, data in Table II also show that cysteine but not glutathione at a concentration of 20 μmoles/ml inhibits the synthesis of citrulline.

It is known that cysteine interferes with the color reaction given by citrulline, when citrulline is determined by the method of Archibald (1). Therefore, two determinations were made. The first one was estimated directly, to the second was added 2 μmoles of cysteine before citrulline was measured. The second sample did show a diminished amount of citrulline but the results shown in Table II still indicate that the inhibition of citrulline synthesis by cysteine is not due to the interfering effect of cysteine on the color reaction given by citrulline. For further clarification an attempt was made to isolate the products of incubation by means of chromatography, using tertiary butanol: —$NH_3$:methylethyl ketone:water (50:15:50:25) in the first, and secondary butanol:formic acid:water (100:15:25) in the second dimension. The results

TABLE II

Effect of carbamyl-L-cysteine on growth and citrulline synthesis in *E. coli*

(*a*)

| Additions | Cell concentration/ml | | |
|---|---|---|---|
| | 0 minutes | 2 hours | 3 hours |
| Nil | $5\times10^7$ | $7.5\times10^8$ | $1.3\times10^9$ |
| 0.02 *M* S-carbamyl-L-cysteine | $5\times10^7$ | $5\times10^7$ | $5\times10^7$ |
| 0.01 *M* L-cysteine | $5\times10^7$ | $6\times10^8$ | $1.0\times10^9$ |

(*b*)

| Additions | Micromoles citrulline in 30 minutes |
|---|---|
| Nil | 8.0 |
| 0.02 *M* S-carbamyl-L-cysteine | 0.5 |
| 0.02 *M* L-cysteine | 0 |
| 0.02 *M* glutathione | 8.0 |
| 0.02 *M* L-cysteine* | 4.0 |

NOTE: For determination of the effect of growth, the bacteria were grown in nutrient broth – glucose. For citrulline synthesis all tubes contained, in a total volume of 1 ml pH 8.5 tris buffer, 20 μmoles each of carbamyl phosphate and ornithine and 0.1 ml of *E. coli* B/1,5 extract from about $10^{10}$ cells/ml. For citrulline determinations 0.1 ml samples were taken according to the method of Archibald (1).

*Cysteine added after incubation to determine its effect on the color development only.

confirmed that there is virtually no citrulline synthesis in the presence of 20 μmoles of cysteine, or carbamyl cysteine, but there was evidence of carbamyl cysteine formation from cysteine plus carbamyl phosphate.

Experiments were performed to observe if there was a chemical combination between carbamyl phosphate or cyanate and cysteine. The results of two-dimensional paper chromatography (8) indicated the formation of carbamyl cysteine when carbamyl phosphate and cysteine were incubated at 37° C for 15 minutes. When KCNO was used as the carbamyl donor, again there was an indication of carbamyl cysteine formation, but in much lower quantities than when carbamyl phosphate was used.

*Interaction of Glutathione with Cyanate*

In order to explain the reversal of the cyanate-caused inhibition of growth by glutathione, the two molecules were incubated together and attempts were made to observe if there was a chemical reaction between them. It was found that when KCNO was incubated with GSH, the medium became alkaline, indicating either the breakdown of cyanate or the removal of the cyanate ion

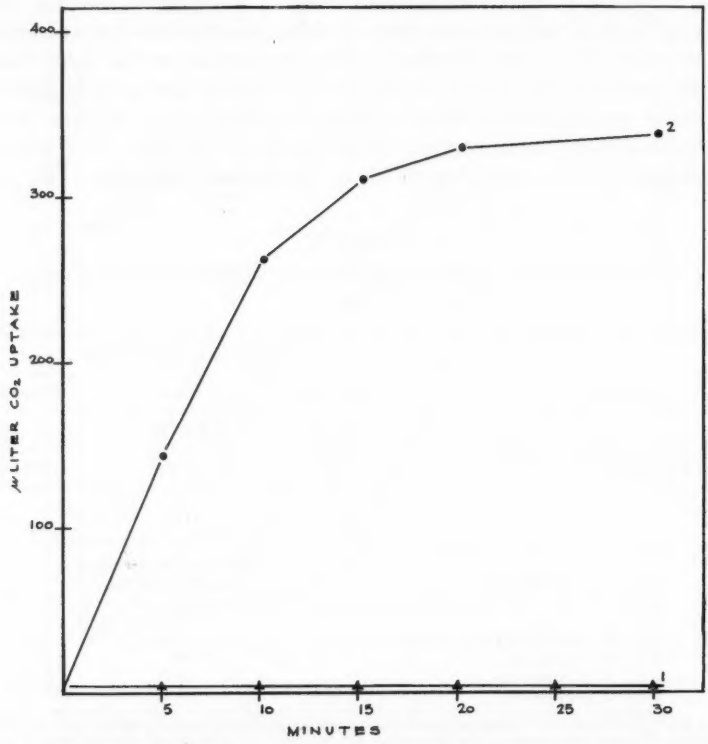


FIG. 5. The effect of GSH on cyanate; 0.02 *M* GSH or KCNO was incubated in a bicarbonate buffer each alone (1) or together (2) in a nitrogen – carbon dioxide atmosphere.

by glutathione. The rate of this reaction can be measured in the conventional Warburg apparatus since for each mole of alkali produced there is 1 mole of $CO_2$ uptake from a nitrogen – carbon dioxide atmosphere. The effects of incubating glutathione with cyanate are shown in Fig. 5.

Since there was no evidence of ammonia production upon mixing the two molecules, and since the mixture of GSH and KCNO could act as inducer of cyanase synthesis (10), it was concluded that the GSH molecule combined with the cyanate ion rather than caused its breakdown.

*Effect of Carbamyl Phosphate and Cyanate on the Formation of β-Galactosidase in E. coli and Staphylococcus pyogenes*

It was felt to be of interest to investigate the effects of carbamyl phosphate and cyanate on a system other than growth. Both compounds were tested for their effect on the formation of the adaptive enzyme β-galactosidase.

Adaptation occurs in staphylococci in a resting cell suspension (2), while in *E. coli*, enzyme synthesis is related to the growth of the cells (7). Table III shows that the synthesis of the enzyme is completely inhibited in staphylococci by both carbamyl phosphate and KCNO, but that in *E. coli*, KCNO inhibits while carbamyl phosphate, if anything, stimulates the synthesis of the enzyme.

Table III also shows that glutathione did not reverse the inhibition of adaptive enzyme formation caused by either carbamyl phosphate or by KCNO in *S. pyogenes*. In the case of *E. coli*, when the growth was restored by GSH, the synthesis of β-galactosidase was also restored, provided KCNO, lactose, and GSH were all added together to the medium.

TABLE III

Effect of KCNO and carbamyl phosphate on β-galactosidase synthesis in *E. coli* and in *S. pyogenes*. The effect of GSH on the synthesis of adaptive enzyme is also shown

| Organisms | Additions | μmoles ONP released | % inhibition |
|---|---|---|---|
| *E. coli* | Nil | 1.8 | Nil |
| *E. coli* | 0.02 *M* KCNO | 0 | 100 |
| *E. coli* | 0.02 *M* CP | 2.1 | Nil |
| *E. coli* | 0.02 *M* KCNO + 0.02 *M* GSH | 2.2 | Nil |
| *S. pyogenes* | Nil | 4.8 | Nil |
| *S. pyogenes* | 0.02 *M* CP | 0.10 | 98 |
| *S. pyogenes* | 0.02 *M* KCNO | 0.40 | 92 |
| *S. pyogenes* | 0.02 *M* KCNO + 0.02 *M* GSH | 0.25 | 95 |
| *S. pyogenes* | 0.02 *M* CO + 0.02 *M* GSH | 0.12 | 98 |

NOTE: β-galactosidase synthesis was determined according to the method of Lederberg, on the basis of the hydrolysis of ONPG to the colored ONP (6).

*E. coli* B/1,5 was grown in nutrient broth + 0.01 mg glucose to a concentration of $3 \times 10^8$ cells/ml. Then were added 5 mg/ml lactose and the stated concentration of CP or KCNO when used.

*S. pyogenes* was grown overnight in nutrient broth + 2 mg/ml glucose. The cells were washed, resuspended at a concentration of 3 mg/ml in a mixture of amino acids, 1 mg/ml glucose, and 10 mg/ml lactose + the stated concentration of CP or KCNO when used.

## Discussion

It has been shown that both carbamyl phosphate and cyanate inhibit the growth of *E. coli*. Since the inhibitory action of the cyanate ion is well known, it is natural to suspect the conversion of carbamyl phosphate to cyanate and so explain the inhibitory action of this compound. However, it does not appear likely that the inhibitory action of carbamyl phosphate is due to its breakdown

to cyanate because preincubation in broth of carbamyl phosphate did not produce the same results as did KCNO when added to logarithmically growing cells. The fact that carbamyl phosphate does not inhibit logarithmically growing bacteria in broth or that its addition to logarithmically growing cells in synthetic medium resulted in an inhibition in less time than it would have required for it to break down to an inhibitory concentration of KCNO certainly suggests a difference of action.

The different action of cyanate and carbamyl phosphate with respect to bacteria in the logarithmic growth phase and lag phase is rather surprising. However, it is possible, in view of the readiness with which carbamyl phosphate forms the inhibitory substance, carbamyl cysteine, that its inhibitory action is due to the formation of this compound intracellularly from the existing free cysteine during the lag phase, but that during the logarithmic phase of growth either there is less free cysteine present or the carbamyl phosphate will be used in the various synthetic processes and has no chance to form the inhibitory compound. The reversibility of the inhibition of both carbamyl phosphate and carbamyl cysteine by GSH or cysteine suggests that a sulphydryl group is involved in the inhibition. The inhibitory action of KCNO could also be explained by the formation of carbamyl cysteine or simply by the affinity of the molecule for SH groups.

The reversal of inhibition by glutathione in the case of KCNO is easy to understand if one accepts that glutathione effectively removes the inhibitory cyanate ion from solution. However, one is hard put to explain in that case why it does not reverse the inhibition of β-galactosidase synthesis in *S. pyogenes* unless this is a matter of the permeability of the GSH molecule, or the behavior of the GSH cyanate complex in *Staphylococcus* is different from its behavior in *E. coli*.

The different behavior of KCNO in the lag phase and in the logarithmic growth phase of *E. coli* might well be a reflection on the level of available SH compounds, which would, in these different phases of the bacterial growth cycle, catalyze the removal of cyanate.

In the case of cyanate, another explanation is also possible. *E. coli* can synthesize the adaptive enzyme cyanase, which breaks down cyanate to ammonia and $CO_2$ (10). This synthesis occurs in the lag phase even when high concentrations of cyanate are present. It can also occur in the logarithmic growth phase, but not in the presence of the higher concentrations of cyanate.

The different action of carbamyl phosphate on growing and non-growing cells extends to the effect of this compound on β-galactosidase synthesis as well. It has no adverse effect on the synthesis of this enzyme in *E. coli*, whose cells are actively growing during the period of enzyme synthesis, but inhibits the synthesis of the enzyme in *S. pyogenes*, an organism that synthesizes β-galactosidase while the cells are at rest.

## Acknowledgment

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# THE FORMATION OF CELLULOSE MICROFIBRILS IN SUSPENSIONS OF ACETOBACTER XYLINUM[1]

J. Ross Colvin and M. Beer[2]

## Abstract

Formation of cellulose microfibrils from precursors elaborated by *Acetobacter xylinum* takes place remote from the cell surface and without an extensive, amorphous, intermediate high polymer. The microfibrils increase in mass by growth only at one or both tips. The rate of microfibrillar growth per bacterial cell at 25° C is constant at 0.1 $\mu$ per minute up to at least 7 minutes, incubation time. New microfibrils may be produced continuously during the incubation period.

## Introduction

The advantages offered by bacterial cellulose for study of the detailed mechanism of formation of cellulose microfibrils have been recognized for some time (11). The filamentous product of *Acetobacter xylinum* is fibrillar 7, 12), similar in solubility (11), crystallinity (11), and composition (1) to cotton cellulose, and, above all, is extrace lular (11, 12). However, the mode of format on of bacterial cellulose microfibrils is still equivocal. From electron microscope observations of pseudoreplicas of slides dipped from cultures of *A. xylinum* at various times, Mühlethaler (12) suggested that the microfibrils were formed remote from the cell surface by crystallization of an extensive, amorphous, capsular, intermediate, high polymer. Interpretation of observations from such preparations is difficult, however, because insufficient washing may remove too little material, and too much washing may damage the specimen. Perhaps because of this, the conclusion that cellulose microfibrils are formed remote from the cell surface has not yet been accepted completely (16). The problem has therefore been reinvestigated using the electron microscope, and incubating cells in a synthetic medium containing only low molecular weight compounds. The suspensions could then be freed of components of the medium by dialysis under conditions which minimize contamination and mechanical disturbance. These conditions also facilitate quantitative study of microfibril formation. The results lead to conclusions which are quite different from previous suggestions.

A preliminary account of this work has been published (5).

## Materials and Methods

In all experiments cellulose-free washed cells of *A. xylinum*, ATCC 10245, were prepared as described by Hestrin and Schramm (11), except that they were not freeze-dried but stored as a suspension in phosphate–citrate buffer (0.01 *M* and 0.003 *M* respectively), pH 6.0. The fresh suspensions contained approximately $10^9$ viable cells per ml determined by the most probable number method (15) or 1–8 mg dry weight of cells per ml in agreement with the results of Hestrin and Schramm (11).

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Formvar films were floated on a 2% glucose, 0.27% $Na_2HPO_4$, and 0.115% citric acid solution, pH 6.0, contained in Petri dishes and equilibrated to the desired temperature (20°, 25°, or 35° C). Drops of an appropriate dilution of cells in the same medium (usually 1 ml of stock suspension of cells to 50 ml of solution) were placed on the film and incubated for 0–7 minutes. In initial experiments, after incubation, 40% formaldehyde was pipetted into the solution under the films to stop metabolic activity by rapid diffusion across the membrane. Later it was found that cell activity was stopped more rapidly without affecting experimental observations by introducing drops of formaldehyde directly into the suspensions on the films. Formaldehyde, glucose, and salts were subsequently removed by repeated replacement of the solution under the films by distilled water. The area of the film carrying the drop was then mounted over a copper electron microscope grid, dried, shadowed with palladium–gold alloy (40:60) at an angle of 15°, and examined.

The number of bacteria relative to the number of microfibrils, and the length of the microfibrils, at each time and temperature, were obtained from sets of 25 adjacent electron micrographs of randomly selected areas on the grids.

Cultures of *A. xylinum* were incubated in 5% gels of carboxymethyl cellulose (CMC) as follows. One-half milliliter of the stock suspension of bacteria was mixed thoroughly with 0.250 g CMC plus 4.5 ml of the glucose–phosphate–citrate solution, pH 6.0, in Petri dishes. The gel cultures were incubated at 26°–28° C. for 20 hours to allow extensive production of microfibrils, and then the cells were killed by flooding with 1% formaldehyde. Most of the cells and all of the CMC were then removed by digestion with 4% NaOH in a boiling water-bath for 10 minutes, followed by centrifugation at 15,000 *g* for 15 minutes. The pellet was redispersed in water and examined in the electron microscope, as above, for the presence of microfibrils.

## Results

Initial photographs confirmed Mühlethaler's conclusions about formation of bacterial cellulose microfibrils remote from the cell surface (Fig. 1). No evidence was found for any association between the bacteria and the microfibrils beyond that expected from projection of a randomly dispersed three-dimensional system on a two-dimensional figure (Fig. 2). Such accidental contacts should be less frequent in dilute preparations and this was indeed found to be the case.

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FIG. 1. Cellulose microfibrils grown in liquid suspension of *A. xylinum*. Incubation time, 90 seconds: 28° C.

FIG. 2. *A. xylinum* cells showing surface contours and the superposition of microfibrils.

FIG. 3. Typical cell membranes and extruded cytoplasm, resulting from mechanical disintegration of *A. xylinum*.

FIG. 4. Short microfibrils showing blunt ends. Note typical pairing of microfibrils.

FIG. 5. Microfibrils formed by *A. xylinum* in a gel of 5% carboxymethyl cellulose.

FIG. 6. Microfibrils of varying length from liquid culture, incubated for 5 minutes.

PLATE I

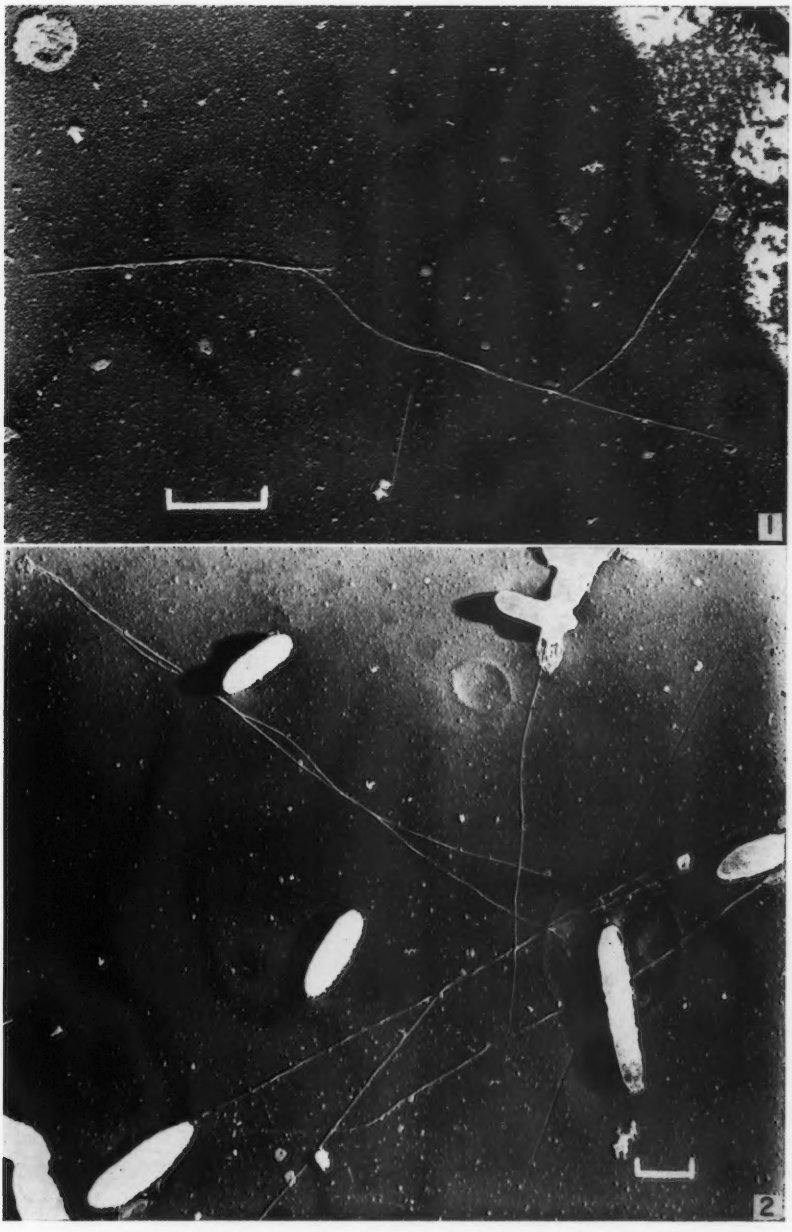

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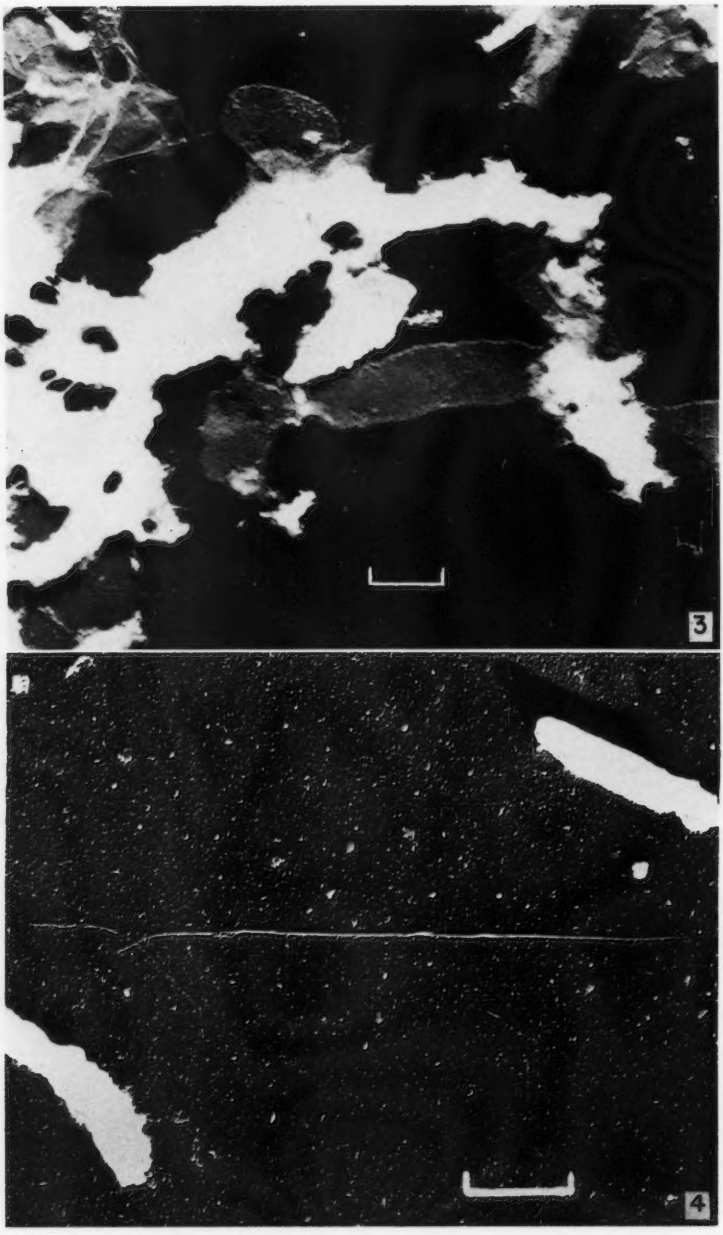
PLATE II

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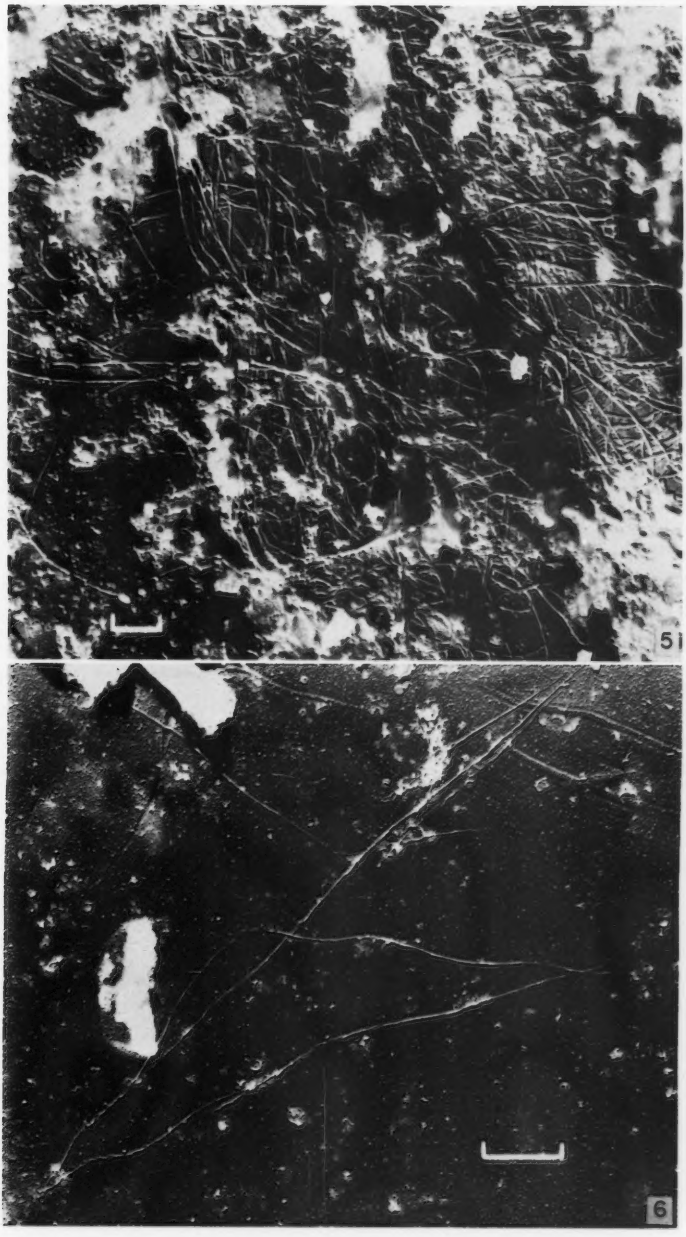
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The surface of the washed cells possessed a smooth or slightly pebbly texture without any suggestion of fibrous material (Fig. 2). Cell ghosts, obtained by sonic disruption of washed cells or by mechanical disintegration showed no fibrillar material within the wall or on its inner surface (Fig. 3). Mühlethaler's (12) observations of flattened ends and dense portions of the bacterial protoplast were confirmed.

The following results indicated that no high polymeric substance is involved as an extracellular intermediate in bacterial cellulose synthesis. Without exception, cells from thoroughly dialyzed cultures exhibited no capsular material although microfibrils were found in abundance (Fig. 2). Autoradiographs of chromatograms (pyridine–ammonia–water 6:2:1) of culture media containing glucose uniformly labelled with $C^{14}$ failed to show any evidence of a water-soluble polysaccharide ($<0.1$ $\mu$g/ml), confirming the results of Hestrin and Schramm (11). The microfibrils themselves were free of amorphous encrusting material and clean and sharp in outline. Most microfibrils occurred in pairs or higher multiples, but occasionally single threads were also observed. Most microfibrils were tapered at the ends (Figs. 1, 2, 6) although rare exceptions were noted (Fig. 4). No fraying of the ends of the microfibrils was observed at any time.

The above observations indicated that a high polymer is not an intermediate in the synthesis of bacterial cellulose and that the microfibrils are formed remote from the cell surface. If this is so, growth of bacterial cellulose microfibrils should not be hindered by the presence of a neutral gel which holds the cells stationary but allows free diffusion of low molecular weight material. Conversely, a stiff gel composed of a chemically similar high polymer would be expected to impede or prevent movement of the supposed intermediate thereby partially or totally inhibiting microfibril formation. Carboxymethyl cellulose at 5% concentration forms such a gel in which cells of *A. xylinum* are held completely immobile (no Brownian motion) but in which very long cellulose microfibrils are readily formed (Fig. 5). No evidence for any inhibition of microfibril formation by the polymer was observed. This result is clearly consistent with the postulate of a low molecular weight intermediate diffusing from the cells to the tips of long growing microfibrils but would be difficult to explain by the notion of an extensive, high-polymeric amorphous phase, sloughed off from the surface of the organism.

During preliminary experiments (5) a correlation between the mean length of microfibrils and incubation time was observed, with no corresponding increase in diameter of the microfibrils. This result was confirmed and indicates that the microfibrils grow only at the tip(s). Quantitative determinations of the mean rate of growth of the microfibrils per bacterium at 25° C were made and the data are summarized in Fig. 7. After a possible induction period of 15–30 seconds the average rate of growth of microfibrils per bacterial cell is constant at 0.1 $\mu$/minute up to 7 minutes. This rate of linear growth corresponds to the addition to a microfibril of approximately $10^3$ glucose residues per bacterial cell per second in reasonable agreement with the mass estimates

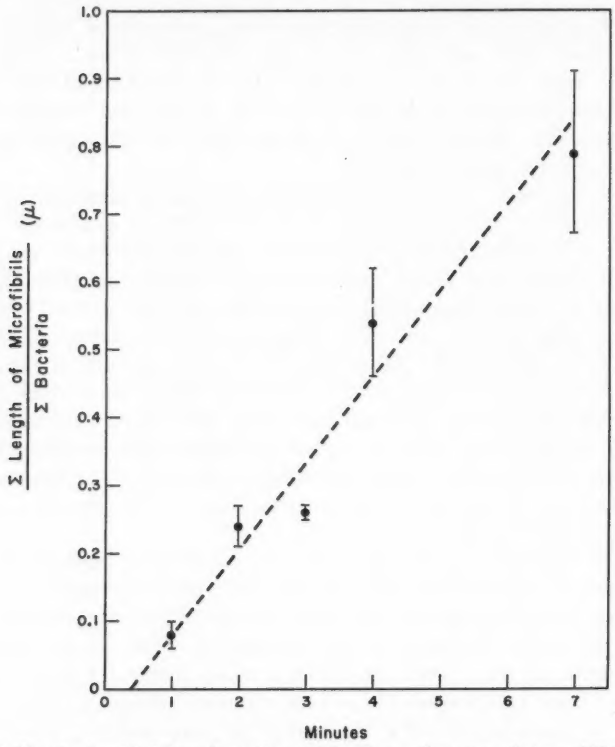


FIG. 7. Total microfibril length per bacterial cell as a function of time; 25° C. Points with their associated lines indicate means ± the standard deviation.

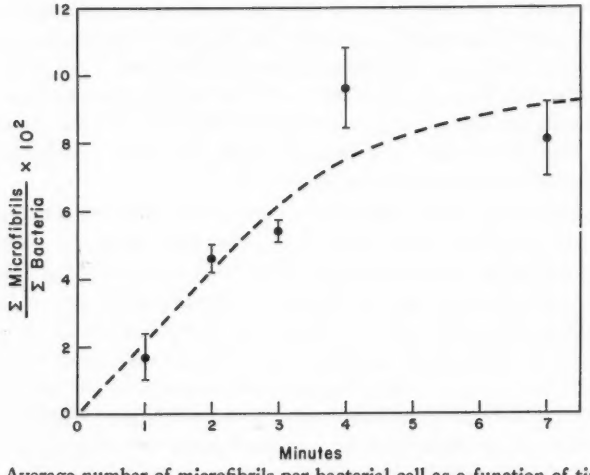


FIG. 8. Average number of microfibrils per bacterial cell as a function of time; 25° C. Points with their associated lines indicate means ± the standard deviation.

of Hestrin and Schramm (11). Presumably the rate declines from this maximal value when the supply of one or more metabolites becomes limiting (11). In addition, Fig. 7 shows how the standard deviation of the average microfibril length per bacterial cell tends to increase as incubation time increases, reflecting the growing heterogeneity caused by the continuing formation of new microfibrils among the old as cultures age (Fig. 6). The ratio of number of microfibrils to number of bacterial cells in a suspension increases up to about five minutes' incubation time, after which it appears to level off (Fig. 8). Although the data are only rough, this may mean that as the total concentration of existing microfibrils increases in a culture, the probability of attachment of a precursor molecule to an existing microfibril is much greater than that of its incorporation into the nucleus of a new microfibril.

To avoid confusion, it is important to note that only the rate of growth of microfibrils measured in terms of total increase in length per bacterial cell is approximately constant with time but not the rate of growth (as linear increase) of individual microfibrils. The over-all reaction is therefore not autocatalytic.

Similar results were obtained for cultures incubated at 35° C. No appreciable growth of microfibrils took place in cultures at 20° C.

## Discussion

The above results corroborate previous suggestions that the cellulose produced by *A. xylinum* is completely extracellular, and they also confirm the previous conclusion that cellulose microfibrils are formed remote from the bacterial cell surface. The conclusion that formation of bacterial cellulose is independent of the cell surface is confirmed by recent reports of cellulose synthesis in supplemented fractionated homogenates (4, 8, 9) and in ultrafiltered ethanol extracts of active cultures (6). Recent studies of the formation of green plant cellulose remote from the cytoplasmic membrane indicate that bacterial cellulose is not unique in this respect (2, 3, 14).

In contrast to previous reports, no evidence was found either by the electron microscope or autoradiography of chromatograms of the culture medium for an extensive amorphous polymeric substance postulated as an intermediate in cellulose synthesis. The earlier conclusions may be attributed to unsuspected contamination of the pseudoreplicas of culture samples.

The above results also show that the bacterial cellulose microfibrils increase in mass only by growth at one or both tips. A glucose residue (or a low polymer of glucose) is added successively to the ends of the $\beta$-glucosan chains already incorporated in the microfibril, rather than deposited in a new layer on the outside surface. This, in turn, implies that polymerization of glucose into the chain and orientation of the added residue in the crystalline portions of the microfibril are nearly simultaneous events. Since less than 30 seconds is required to establish a steady-state concentration of the precursor in the medium in active cultures, it is likely that no more time would be necessary to remove nearly all of this intermediate from a culture after inhibition of cell metabolism,

provided that the polymerizing enzyme was not also inhibited (6). The short time required to establish the steady state also suggests that the absolute concentrations of the free precursor in active cultures are low.

The significance of the association of the microfibrils is difficult to evaluate at present. Whether it is due to more or less random accidental alignment under the influence of lateral van der Waals forces or to side-by-side growth cannot be determined yet. However, its importance for the correlation of the length of microfibrils with time of growth and for the determination of microfibril diameters is obvious.

These observations cannot throw much light on the nature of the extracellular intermediate beyond indicating that it is a compound of relatively low molecular weight. The suggestion of Schramm, Gromet, and Hestrin (10, 13) that the immediate precursor is a derivative of a hexose phosphate ester is consistent with the above data but not proved by them.

Hestrin and Schramm (11) have stressed that the fine structure of the polymerization product of isolated washed cells and that from native cultures are identical. They have also observed that bacteria may continue to synthesize cellulose gels of increasing strength as incubation time goes on. Both observations are consistent with the concepts presented here.

The picture of cellulose microfibril formation sketched above is sharply at variance with that tentatively accepted hitherto but is completely consistent with present information. How the low molecular weight activated derivative of glucose is formed within the bacterial cell, is released into the medium, and is finally incorporated into the tip of the microfibril remains to be studied.

## Acknowledgments

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# NUTRITION AND METABOLISM OF MARINE BACTERIA

## X. THE GLYOXYLATE CYCLE IN A MARINE BACTERIUM[1]

R. A. MacLeod,[2] Aiko Hori,[3] and Sylvia M. Fox


### Abstract

Extracts of a species of marine bacterium have been shown to contain isocitratase and malate synthetase when cells were grown in a medium in which acetate was the sole source of carbon and energy. Neither enzyme could be demonstrated in extracts prepared from cells grown in a nutrient broth, yeast extract medium.


Isocitratase, which converts isocitrate to glyoxylate and succinate (11), and malate synthetase, which condenses acetyl coenzyme A and glyoxylate to form malate (14), together make up what has been termed the glyoxylate bypass in the tricarboxylic acid cycle (4). The operation of this bypass, serving as a source of the four-carbon compounds necessary for the continued operation of the tricarboxylic acid cycle, provides an explanation for the capacity of cells to grow in a medium containing acetate as the sole source of carbon and energy.

Both isocitratase and malate synthetase have been shown to be present in a wide variety of microorganisms (6). In view of the possibility that terrestrial bacteria have evolved from marine bacteria (7) it was of interest to know if these enzymes are also present in a bacterium of marine origin. In this report a species of marine bacteria which has been found to contain all of the enzymes of the tricarboxylic acid cycle (9) is shown also to be capable of forming the key enzymes of the glyoxylate bypass.

### Experimental

The marine bacterium used in this study is a species of either a *Pseudomonas* or a *Spirillum* and has been designated B-16 in this and other communications. The organism was isolated from marine clams and has been shown to have a highly specific requirement for $Na^+$ for growth. Its marine origin coupled with its need for $Na^+$ serves to distinguish this bacterium from terrestrial species (7).

Two growth media were used in this study. One, referred to as the acetate medium, contained the following (in grams per liter): sodium acetate, 20; NaCl, 12.7; $MgCl_2.6H_2O$, 5.32; $(NH_4)_2SO_4$, 1.0; KCl, 0.73; $K_2HPO_4$, 0.105; $KH_2PO_4$, 0.105; and $FeSO_4(NH_4)_2SO_4.6H_2O$, 0.035. The other contained nutrient broth 8, and yeast extract 5, in place of the sodium acetate.

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The methods used to cultivate and harvest the cells and to prepare sonic extracts have been described (8). Growth on the acetate medium was slower than on the nutrient broth, yeast extract medium. With the former, 40–45 hours', and with the latter, 18 hours' incubation were required to obtain a good yield of cells.

Except where indicated, cells were washed and suspended in 0.02 *M* phosphate buffer containing 0.5 mg per ml of glutathione.

Isocitratase activity was measured in an assay system which was essentially that of Smith and Gunsalus (13) modified to provide conditions more nearly optimum for the enzyme from this organism. The assay was carried out in 15-ml Warburg flasks attached to the manometers of a Warburg respirometer. Each flask contained Tris[4] buffer pH 7.5, 125 $\mu$moles, DL-isocitrate (Tris salt) 40 $\mu$moles, $MgCl_2$ 6 $\mu$moles, cysteine 4 $\mu$moles, and sonic extract of the cells. The total volume was 3 ml, the gas phase $N_2$, the temperature 25°, and the incubation time 60 minutes (unless otherwise indicated). The reaction was stopped by addition of 0.2 ml of 80% trichloracetic acid, the mixture was centrifuged at 50,000 *g* for 30 minutes to remove protein and the supernatant analyzed for glyoxylate formed. The results reported were corrected for glyoxylate formation in flasks identical except for the addition of substrate to the reaction mixture after the reaction had been stopped by trichloracetic acid.

Glyoxylate was measured as the 2,4-dinitrophenylhydrazone according to the procedure of Smith and Gunsalus (13) using glyoxylic acid (California Foundation) as the standard. Although the latter was a "purified" rather than a "chemically pure" reagent, it gave a linear relation between optical density and concentration in the colorimetric test applied and hence was considered satisfactory for the purposes intended. Glyoxylate was identified on paper chromatograms as its 2,4-dinitrophenylhydrazone using the ammonia, butanol solvent system of Cavallini *et al.* (1). Reversal of the isocitratase reaction was shown by demonstrating isocitrate formation from glyoxylate and succinate. Isocitrate was detected chromatographically using two solvent systems: *n*-butanol, isobutanol, formic acid, $H_2O$ and isoamyl alcohol, formic acid, $H_2O$ (3) as well as spectrophotometrically by following TPN reduction at 340 m$\mu$ under conditions optimum for the activity of the isocitric dehydrogenase present in the extract (9, 10).

Malate synthetase was detected by demonstrating malate formation from acetyl CoA and glyoxylate in the presence of cell-free extracts of the organism. Acetyl CoA was generated from acetyl $PO_4$ and CoA by the action of phosphotransacetylase (Worthington Biochemical Corp.). Malate was demonstrated chromatographically on paper using the solvent system *n*-butanol, isobutanol, formic acid, $H_2O$ (3) and spectrophotometrically by following TPN reduction at 340 m$\mu$ under conditions known to be optimum for the malic dehydrogenase present in the extracts (7).

[4]ABBREVIATIONS USED: Tris, tris(hydroxymethyl)aminomethane; TPN, triphosphopyridine nucleotide; CoA, coenzyme A; ATP, adenosine triphosphate.

## Results and Discussion

*Demonstration of the Presence of Isocitratase*

No evidence of the presence of isocitratase could be obtained in extracts of cells grown in a nutrient broth yeast extract medium. When the cells were grown in a medium containing acetate as the sole source of carbon, extracts prepared from the cells contained the enzyme. This is in accord with observations made previously with terrestrial bacteria that the enzyme forms adaptively only under conditions where the net formation of $C_4$ compounds from acetate is necessary for growth (5, 12).

The enzyme was detected by the capacity of the extract to form glyoxylate from isocitrate. Glyoxylate was identified both by the position of its 2,4-dinitrophenylhydrazone on a paper chromatogram and by the characteristic red-brown color which developed at the spot on spraying with alkali. The 2,4-dinitrophenylhydrazone of another keto acid, pyruvate, was also extracted from the reaction mixtures, both in the presence and absence of isocitrate. Since this compound could interfere with the determination of glyoxylate by the method used, it was of interest to know if the pyruvate level varied in the reaction mixture upon the addition of substrate. When the optical density readings at 490 and 540 m$\mu$ of a system producing glyoxylate from isocitrate were corrected for endogenous color formation, the ratio of corrected optical densities was 2.03. The 490/540 ratio of the 2,4-dinitrophenylhydrazone of glyoxylate is 1.9, in good agreement with this value, whereas those of the 2,4-dinitrophenylhydrazones of pyruvic and $\alpha$-ketoglutaric acid are 1.1 and 1.0 respectively (13). It would thus appear that the pyruvate level does not vary when the substrate is present and that the color to which it gives rise can be corrected for satisfactorily with the endogenous control.

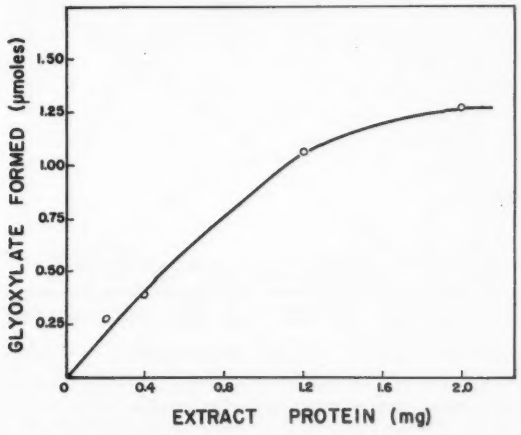


FIG. 1. Glyoxylate formed from isocitrate in response to increasing concentrations of cell-free extract of marine bacterium B-16. Glyoxylate, measured as its 2,4-dinitrophenylhydrazone, was determined after an incubation period of 60 minutes under conditions described in the Experimental section.

Glyoxylate formation from isocitrate in response to increasing concentrations of extract protein is shown in Fig. 1. The response obtained is not linear at higher extract concentrations as might be expected when a crude extract is used and a number of reactions compete both for the substrate and the product formed. Succinate could not be detected in the reaction mixture. Since the reverse reaction, succinate and glyoxylate forming isocitrate, was readily demonstrated, it is reasonable to conclude that succinate must have formed from isocitrate and was further metabolized by the crude extract used.

*Reversibility of Isocitratase*

Advantage was taken of the fact that the extract of this marine bacterium contains a powerful isocitric dehydrogenase if it is prepared and tested under appropriate conditions (9, 10). Formation of isocitrate from a combination of glyoxylate and succinate was indicated spectrophotometrically by following TPN reduction at 340 m$\mu$ under conditions optimum for the activity of the isocitric dehydrogenase in the extract (Fig. 2). Glyoxylate and succinate tested separately were without activity. Malate, which can also cause TPN reduction, was likewise ineffective due to the absence of added $K^+$, a requirement for the activity of the malic dehydrogenase present in the cells (8). Isocitrate formed from glyoxylate and succinate in this system was identified by paper chromatography.

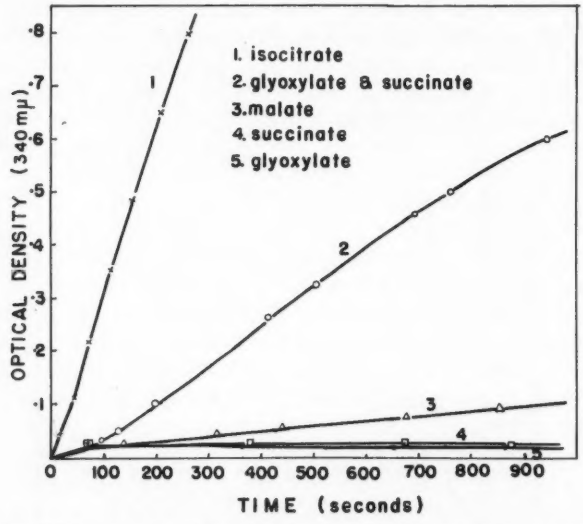


FIG. 2. Spectrophotometric evidence for the reversal of the isocitratase reaction, showing capacity of glyoxylate and succinate together to cause TPN reduction under conditions optimum for the activity of the isocitric dehydrogenase present in the bacterial extract. Complete system: Tris buffer pH 7.5, 250 $\mu$moles; $MgCl_2$, 50 $\mu$moles; TPN, 1 $\mu$mole; cell-free extract $\simeq$ 15 mg protein. Substrates added, where indicated, in following amounts: glyoxylate, 10 $\mu$moles; succinate, 20 $\mu$moles; *dl*-isocitrate, 20 $\mu$moles; malate, 20 $\mu$moles. Total volume, 3 ml. Temperature 25°.

*Malate Synthetase*

Spectrophotometric evidence for the presence of malate synthetase in the marine bacterial extract is presented in Fig. 3. Under conditions giving rise to acetyl CoA, the presence of glyoxylate in a system containing the extract caused TPN reduction after a suitable incubation period when the pyridine nucleotide coenzyme for the malic dehydrogenase of this organism was added to the system. Under the conditions used, a 60-minute incubation period prior to the addition of TPN was the minimum required to cause a reasonable rate of reduction. Neither glyoxylate nor acetyl phosphate alone produced a change in optical density but malate was effective. That malate was indeed the product of the reaction was indicated by paper chromatography of an ether extract of the acidified reaction mixture.

No evidence of malate synthetase activity was obtained in enzyme extracts from cells grown on nutrient broth yeast extract medium. In other microorganisms examined, malate synthetase is much less dependent on growth substrate than isocitratase (6). Although, in this experiment, phosphotransacetylase was used to generate acetyl CoA, an acetate-activating system requiring ATP and CoA can be demonstrated in extracts of acetate-grown cells of this organism (9).

It is clear from these experiments that the key enzymes of the glyoxylate bypass can be demonstrated in this species of marine bacterium under conditions which would be expected to require their presence.

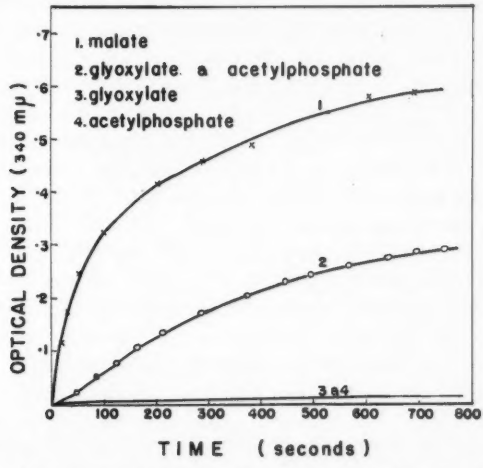


FIG. 3. Spectrophotometric evidence for the presence of malate synthetase in cell-free bacterial extracts of marine bacterium B-16, showing capacity of glyoxylate plus acetyl phosphate to cause TPN reduction under conditions optimum for the malic dehydrogenase present. Complete system: Tris buffer, pH 7.5, 150 μmoles; Tris glyoxylate, 25 μmoles; acetylphosphate, 25 μmoles; CoA, 1.25 μmoles; phosphotransacetylase, 0.2 mg; $K_2SO_4$, 33 μmoles; $MgCl_2$, 100 μmoles; extract ≃ 2.5 mg protein; Tris malate where indicated, 15 μmoles. Total volume, 2.5 ml. Incubated 1 hour at room temperature in 3-ml cuvettes. Then 0.5 μmoles TPN and extract ≃ 2.5 mg protein added and change in optical density recorded.

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# FACTORS INFLUENCING THE GROWTH OF MICROCYSTIS AERUGINOSA KÜTZ. EMEND. ELENKIN[1]

A. ZEHNDER[2] AND P. R. GORHAM

## Abstract

Yields of unialgal *M. aeruginosa* NRC-1 were increased from 685 mg dry wt./liter in 20 days to 1500 mg dry wt./liter in 7 days by increasing the concentrations of $NaNO_3$, $K_2HPO_4$, and $MgSO_4$ in the medium of Fitzgerald *et al.* Within the concentration range that was favorable for growth the balance between these three salts was not especially critical. A low potassium tolerance was noted that appears to depend on the ratio of sodium to potassium in the medium. With the media used, growth was not limited by deficiencies of minor elements, iron, chelation, or any of 10 different vitamins, but was promoted by soil extract. The optimum temperature was approximately 28° C; the optimum pH range was between 8 and 11. Vigorous aeration and agitation promoted growth by insuring better gas exchange and more efficient utilization of light and also, perhaps, by helping to relieve an inhibition caused by continuous leakage and/or lysis of the algal cells. Lysis was promoted by an excess of sodium (or deficiency of calcium) plus other unknown factors. Rapid growth with reasonably high yields was obtained under conditions which are suitable for large-scale culture operations.

## Introduction

Species of planktonic blue-green algae such as *Microcystis aeruginosa* Kütz. emend. Elenkin are frequently found in heavy concentrations (waterblooms) in eutrophic lakes, reservoirs, or slow-moving streams. Although waterblooms appear to develop quite rapidly in nature, comparatively few of the blue-green species involved have been successfully cultured in the laboratory and growth rates have generally been low (15). Canabaeus (9) was one of the first to report the successful culture of *Microcystis aeruginosa*. She obtained growth under anaerobic conditions. In 1950, Gerloff, Fitzgerald, and Skoog (15) reported the successful isolation, purification, and culture of a number of blue-green algae, including *M. aeruginosa*. They grew their algae under aerobic conditions using a modified Chu No. 10 medium (10). Gerloff *et al.* studied the mineral nutrition of their strain of *M. aeruginosa* in some detail (16, 17). They reported a maximum yield of 399 mg dry wt./liter (17) in 20 days. They suggested (16), however, that additional studies of nutrient solutions and culture conditions should be carried out to determine whether or not these figures represented the maximum attainable population density. Olson (32), meanwhile, isolated other strains of *M. aeruginosa* and obtained heavy growths, comparable with blooms observed in the field. He used modified Gericke's solution with added bone meal, but gave no indication of growth rates.

In two preceding papers dealing with the toxicity of *M. aeruginosa* NRC-1, the culture conditions that were employed for rapid growth were described briefly (8, 23). Before these toxicity studies were carried out, however, factors

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Issued as N.R.C. No. 6036.
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influencing the growth of the alga were investigated with the object of attaining the highest possible growth rates and yields and of developing suitable methods for large-scale culture. This paper deals with that investigation. The medium of Fitzgerald *et al.* (12) was used as a basis for studying the mineral nutrition. The effects of varied pH, temperature, and aeration were examined and comparisons were made of growth on nutrient solutions developed for other species of Myxophyceae.

## Materials and Methods

The nomenclature and methods used to obtain *M. aeruginosa* NRC-1 in unialgal culture have been described (23). As previously stated, the alga originally had large globose colonies (Fig. 1A) with diffluent sheaths, but after ultraviolet irradiation and many transfers, the growth habit changed. It now consists mostly of single and paired cells (Fig. 1B) and occasional small colonies with diffluent sheaths (Figs. 1B, 1C). The cells have pronounced pseudovacuoles and are 4 to 5 $\mu$ in diameter.

In most experiments the alga was grown on a small scale in 50-ml Erlenmeyer flasks that were stoppered with cotton. Each flask contained 25 ml of medium and was inoculated with 1 ml (0.5 to 1.5 mg dry weight) of a well-developed suspension from a culture that had been grown under similar conditions to those used for experimentation. Cultures were continuously shaken on a rotary shaker operated at 30 r.p.m. in a room maintained at $21 \pm 1°$ C or $27 \pm 1°$ C as indicated. They were continuously illuminated from above by "White" fluorescent lamps (Fig. 1D). Light intensities varied in different experiments from 1000 to 4000 lux.

For temperature experiments, 1-liter Erlenmeyer flasks containing 700 ml of medium plus 25 ml of inoculum were used. These were incubated at different temperatures under fluorescent light of approximately 2000 lux and were shaken by hand once or twice daily.

A modified "tower-type" fermentor (29) was employed at room temperature for large-scale culture (Figs. 1E, 1F). This consisted of a cylinder of 3.5-liter capacity fitted with a medium-porosity sintered-glass plate, a sampling outlet at the bottom, and ports at the top for the addition by gravity of inoculum, sterile water, or culture medium. Initially the top was plugged with cotton (Fig. 1E) but this often became wet with foam and was later replaced (21) by a cold-finger condensor in a ground-glass joint (Fig. 1F) that minimized evaporation, checked excessive foaming, and prevented additional contamination by bacteria or molds. The fermentor was filled with 2.6 liters of medium plus 100 ml of inoculum and aerated at 1000 cc/minute with dried, filtered, compressed air that was metered at the inlet. Two fermentors mounted side by side were continuously illuminated by twin 40-w "White" fluorescent lamps with reflectors located on opposite sides (Fig. 1E). On the side next to the lamps the intensity was 6500 lux; on the opposite side it was 4500 lux. Cultures were sampled by first adding water to make up for evaporative losses and then all but 100 ml could be withdrawn

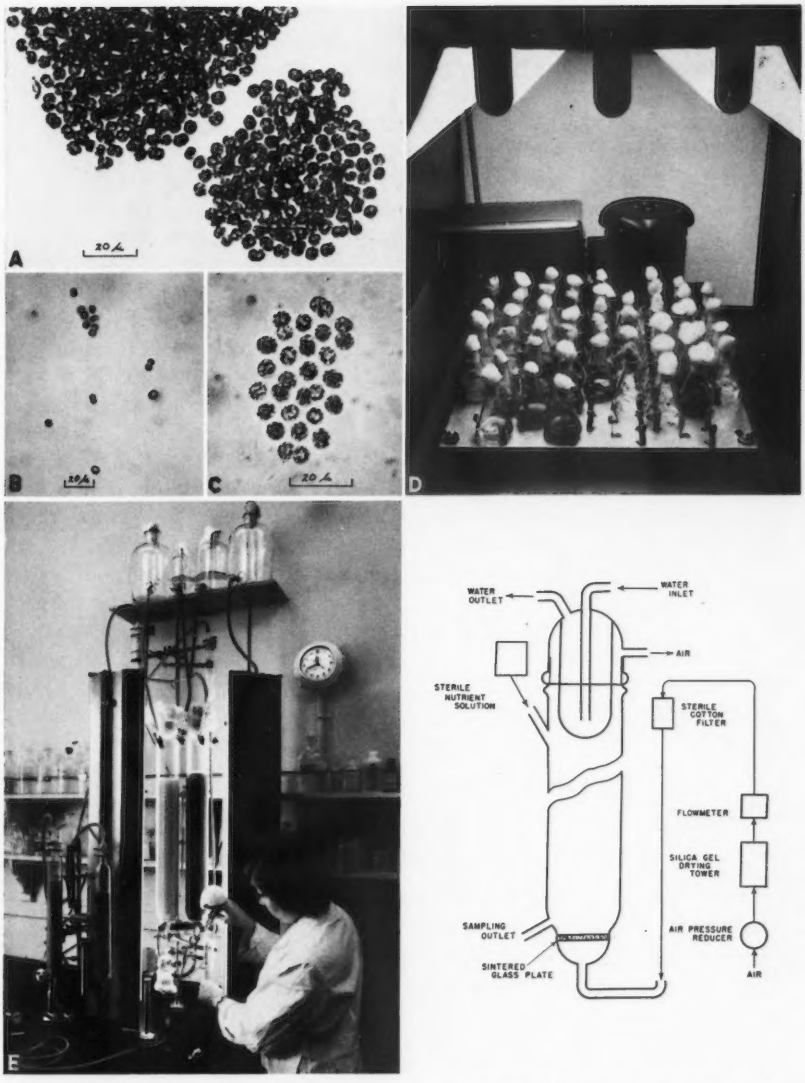



FIG. 1. (A) *Microcystis aeruginosa* Kütz. emend. Elenkin. Globose colony as originally isolated. (B, C) *M. aeruginosa* NRC-1. After many transfers only single cells, paired cells, and small colonies. (D) Continuously illuminated flask cultures on rotary shaker. (E) Tower-type fermentors continuously illuminated from opposite sides. (F) Diagram of tower-type fermentor with cold-finger condensor top and auxiliary equipment.

through the cotton-stoppered bell at the bottom. Fresh culture solution could then be added from the reservoir at the top. When reservoirs for water or medium became empty they could be replaced by aseptic exchange of ball joints used for connecting the rubber hoses with the fermentor. Subculturing could thus be continued for long periods.

An adaptation of the fermentor for even larger-scale culture was also tested at room temperature. This consisted of a 9-liter Pyrex bottle containing 6 liters of medium plus 250 ml of inoculum that was aerated at 4200 cc/minute through a medium-porosity fritted-glass filter stick inserted through the neck. The bottle was illuminated by three, evenly spaced, 32-w "Circline" "White" fluorescent lamps that surrounded it.

Aseptic technique was employed throughout. Fermentors were sterilized by flowing steam. Other apparatus and media were autoclaved or dry-sterilized. Culture media were prepared with demineralized water and reagent grade chemicals. Light intensities were measured with a Weston meter equipped with two photocells that were not color-corrected. The photocells were held at the same height and in the same plane as the surface of the culture solution in flasks. Since the fermentors were illuminated unequally from different directions, exact integration with the light meter was impossible. Intensities were arbitrarily expressed as the sum of the values obtained with the photocells facing the lamps on opposite sides. Growth was measured by dry weight determinations on aliquots of suspension that were corrected for the weight of salts in the medium (3).

The composition of modified Fitzgerald's medium is given in Table I. The modification consisted of the addition of minor elements. The same concentration of Gaffron's minor element solution (Table I) was substituted for Hoagland's A-Z minor element solution (22) used earlier by Gerloff *et al.* (16). The composition of other media used or referred to in this study is also given in Table I for comparison.

## Results

*Minor Elements; Growth Factors*

Gerloff, Fitzgerald, and Skoog (16, 17) used 0.04× diluted Hoagland's A-Z minor element solution in their medium for *Microcystis* but later omitted it without comment (12). Equally good yields of *M. aeruginosa* NRC-1 were obtained when minor elements were omitted for two successive transfers or when 0.04× to 0.08× diluted Hoagland's A-Z, Gaffron's, or Arnon's A5 (5) (Table I) minor element solutions or Arnon's A5 solution plus 0.146 mg/liter $Co(NO_3)_2.6H_2O$ were tested in Fitzgerald's medium. The more concentrated minor element solution used by Allen (2) proved toxic under the same conditions. The addition of 1 mg/liter of ethylenediaminetetraacetic acid (EDTA) to mod. Fitzgerald's medium had no significant effect upon growth as long as the concentration of minor elements was low. When the concentration of Gaffron's minor element solution was increased in stages from 0.04 to 0.64 ml/liter the highest concentration was toxic in the absence but not

TABLE I

Composition of media used for cultivation of Myxophyceae expressed in mg/liter

| | Mod. Chu 10 (10) | Mod. Fitzgerald (12) | No. 11 | Kratz–Myers C (27) | Kratz–Myers D (27) | Allen–Arnon (3) | No. 32 |
|---|---|---|---|---|---|---|---|
| $NaNO_3$ | | 124 | 496 | | 1000 | | 850 |
| $KNO_3$ | | | | 1000 | | 2020 | |
| $Ca(NO_3)_2.4H_2O$ | 58 | | | 25 | 10 | | |
| $K_2HPO_4.3H_2O$ | 13 | 13 | 39 | 1310 | 1310 | 456 | 137 |
| $MgSO_4.7H_2O$ | 25 | 25 | 75 | 250 | 150 | 246 | 125 |
| $CaCl_2.2H_2O$ | | 36 | 36 | | | 74 | 43 |
| NaCl | | | | | | 232 | |
| $Na_2CO_3$ | 20 | 20 | 20 | | | | 20 |
| $Na_2SiO_3.9H_2O$ | 58 | 58 | 58 | | | | 116 |
| Na-citrate $.2H_2O$ | | | | 165 | | | |
| $Fe_2(SO_4)_3.6H_2O$ | | | | 4 | 4 | | |
| EDTA | | | 1 | | 50 | | |
| Ferric citrate | 3 | 3 | 6 | | | | |
| Citric acid | 3 | 3 | 6 | | | | |
| Fe (as EDTA-complex) | | | | | | 4 | |
| Fe (as Versene-diol-complex) | | | | | | | 0.75 |
| Gaffron's minor element solution* | 0.04 ml | 0.04 ml | 0.08 ml | | | | 0.08 ml |
| Arnon's A5 minor element solution† | | | | 1.0 ml | | | |
| H5 minor element solution‡ | | | | | 1.0 ml | | |
| Allen and Arnon's minor element solution§ | | | | | | Each part 1.0 ml | |

* Gaffron's (23) minor element solution (in g/liter): $H_3BO_3$ 3.100, $MnSO_4.4H_2O$ 2.230, $ZnSO_4.7H_2O$ 0.287, $(NH_4)_6Mo_7O24.4H_2O$ 0.088, $CuSO_4.5H_2O$ 0.125, $Co(NO_3)_2.6H_2O$ 0.146, $Al_2(SO_4)_3$ $K_2SO_4.24H_2O$ 0.474, $NiSO_4(NH_4)_2SO_4.6H_2O$ 0.198, $Cd(NO_3)_2.4H_2O$ 0.154, $Cr(NO_3)_2.7H_2O$ 0.037, $V_2O_4(SO_4)_3.16H_2O$ 0.035, $Na_2WO_4.2H_2O$ 0.033, KBr 0.119, KI 0.083.

† Arnon's A5 (5) minor element solution (in g/liter): $H_3BO_3$ 2.86, $MnCl_2.4H_2O$ 1.18, $ZnSO_4.7H_2O$ 0.222, $MoO_3$ (85%) 0.018, $CuSO_4.5H_2O$ 0.079.

‡ H5 (27) minor element solution (in g/liter): $MnCl_2.4H_2O$ 1.44, $ZnSO_4.7H_2O$ 8.82, $MoO_3$ 0.71, $CuSO_4.5H_2O$ 1.57, $Co(NO_3)_2.6H_2O$ 0.49.

§ Allen and Arnon's (4) minor element solution: part A (in g/liter): $H_3BO_3$ 2.86, $MnSO_4.4H_2O$ 2.03, $ZnSO_4.7H_2O$ 0.222, $MoO_3$ 0.15, $CuSO_4.5H_2O$ 0.079. Part B (in g/10 liters of 0.1 *N* $H_2SO_4$): $Co(NO_3)_2.6H_2O$ 0.494, $NiSO_4.6H_2O$ 0.448, $Cr_2(SO_4)_3.K_2SO_4.24H_2O$ 0.960, $NH_4VO_3$ 0.230, $Na_2WO_4.2H_2O$ 0.179, $TiO(C_2O_4K)_2.2H_2O$ (prepared by dissolving 0.737 g of $TiO(C_2O_4K)_2.2H_2O$, precipitating with $NH_4OH$, filtering, dissolving precipitate in 0.1 *N* $H_2SO_4$ and adding to the rest of part B[4]).

in the presence of EDTA. The final yield was not affected by the high concentration of chelated micronutrients. In subsequent experiments, 0.04 or 0.08 ml/liter of Gaffron's minor element solution (often with 1 mg/liter EDTA) was generally used.

The effects of adding lake water, vitamins, yeast extract, or soil extract to mod. Fitzgerald's medium were examined. Water from the same lake from which *M. aeruginosa* NRC-1 had been isolated produced only limited growth by itself (Table II). When used to supply 50% of the water in mod. Fitzgerald's medium it produced yields that surpassed those of the control by only the small amount observed when used by itself. A supplement of 10 vitamins had no effect upon growth, while the addition of 0.04% Difco yeast extract proved toxic. The addition of 8% by volume of soil extract resulted in final yields that were significantly higher than the controls. Nevertheless, soil extract was not used routinely because of its unknown and variable composition.

TABLE II

Yields of duplicate cultures grown for 20 days in lake water or modified Fitzgerald's medium with various supplements; 25-ml shake flasks, 2000 to 2500 lux, 20 to 22° C

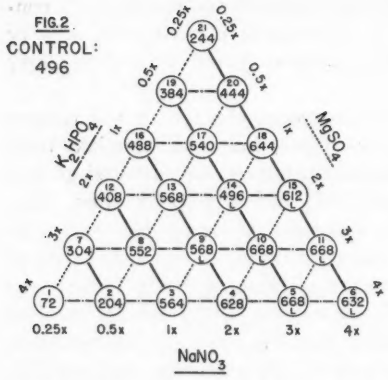
| Medium | Yield, mg dry wt./liter | |
|---|---|---|
| | 1st flask | 2nd flask |
| Lake water | 52 | 64 |
| Mod. Fitz. | 684 | 688 |
| Mod. Fitz. with 50% lake water | 724 | 772 |
| Mod. Fitz. with vitamins* | 668 | 684 |
| Mod. Fitz. with 0.04% Difco yeast extract | 0 | 0 |
| Mod. Fitz. with 8% soil extract† | 835 | 855 |

*Thiamin, riboflavin, pyridoxine, mesoinositol, Ca-pantothenate, niacin, folic acid, *p*-aminobenzoic acid 0.04 mg/liter each, biotin 0.008 mg/liter cobalamin 0.004 mg/liter added to the basic medium after autoclaving.

†Soil extract was prepared by boiling 100 g carbonated soil for 15 to 30 minutes with 500 ml water, cooling, filtering, and making to 250-ml volume (pH 7.0). Carbonated soil consisted of 1300 g dried composted soil mixed with 5 g $Ca(OH)_2$ and 10 g $CaCO_3$.

*Major Elements*

The effects of varying the concentrations and relative proportions of $NaNO_3$, $K_2HPO_4$, and $MgSO_4$ in mod. Fitzgerald's medium from 0.25× to 4× were examined (Figs. 2, 3) after preliminary investigation that indicated that yields were reduced by diluting the concentration of these salts to 0.5×. All other constituents in the 21 different media were the same as in mod. Fitzgerald's. Cultures were grown in duplicate at two light intensities of approximately 4000 and 1000 lux. The yields after 15 days at the higher light intensity are presented in Fig. 2. The highest were in media Nos. 4, 5, 6, 10, 11, 15, and 18, which had 2× to 4× $NaNO_3$, 1× to 4× $MgSO_4$, and 1× to 0.25× $K_2HPO_4$. The maximum yield was 668 mg/liter. This was significantly higher than that of the control in mod. Fitzgerald's medium. The cultures at 1000 lux grew more slowly but after 25 days the final yields were higher (Fig. 3) than those of the cultures grown at 4000 lux for 15 days (Fig. 2). There was no apparent lysis. The highest yields were in media Nos. 5, 10, 11, 14, and 15. The maximum obtained in medium No. 10 was more than twice as great as that of the control.



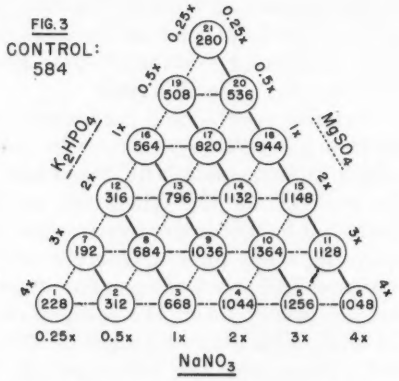




FIG. 2. Average yields of duplicate cultures grown for 15 days at 4000 lux on 21 modifications of Fitzgerald's medium in which the concentrations of the three major salts were varied as shown. In each circle the medium number is given above and the yield in mg dry wt./liter below. L = lysing culture. 50-ml shake flasks, 20 to 22° C.

FIG. 3. Average yields of duplicate cultures grown for 25 days at 1000 lux under conditions otherwise similar to those of Fig. 2.

Initially, all cultures displayed a colonial growth habit, but this gradually changed to a homogeneous suspension of single cells or microcolonies. The cultures with the higher yields tended to maintain the colonial state longer than those with lower yields. With increasing age, cultures were observed to change from blue-green to yellow-green and finally to yellow. Cultures on medium No. 1 were the first to change color followed by the other cultures with 0.25× $MgSO_4$. The cultures with 0.5× and 1× $MgSO_4$ were next to turn. Among these, cultures containing the lower $NaNO_3$ concentrations changed color first. There was no apparent effect of $K_2HPO_4$ concentration on pigmentation. Medium No. 11, in which the algae grew well and remained blue-green longer than in medium No. 10, was adopted for subsequent use as a substantial improvement over mod. Fitzgerald's medium.

*M. aeruginosa* NRC-1 was unable to grow in the absence of combined nitrogen in the medium. This is interpreted as a confirmation of the inability of this species to fix atmospheric nitrogen (37).

*Iron*

Rodhe (35) has emphasized the importance of providing a source of iron that remains in solution under alkaline conditions. He recommends the use of ferric citrate – citric acid autoclaved separately from the rest of the medium. Freshly prepared medium No. 11 without iron has a slight precipitate that tends to disappear when the suspension stands in contact with air. After autoclaving, the precipitate is heavier but the solution becomes clear again upon standing at room temperature. There are downward shifts in pH during storage at room temperature (Fig. 4) that are correlated with the disappearance of precipitate. Kratz and Myers (27) have noted a similar disappearance

of precipitates in their media upon cooling. The iron solution was therefore autoclaved separately and added to the rest of the medium after standing for 24 hours at room temperature.

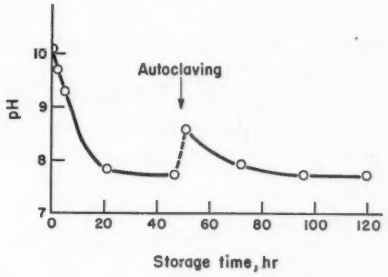
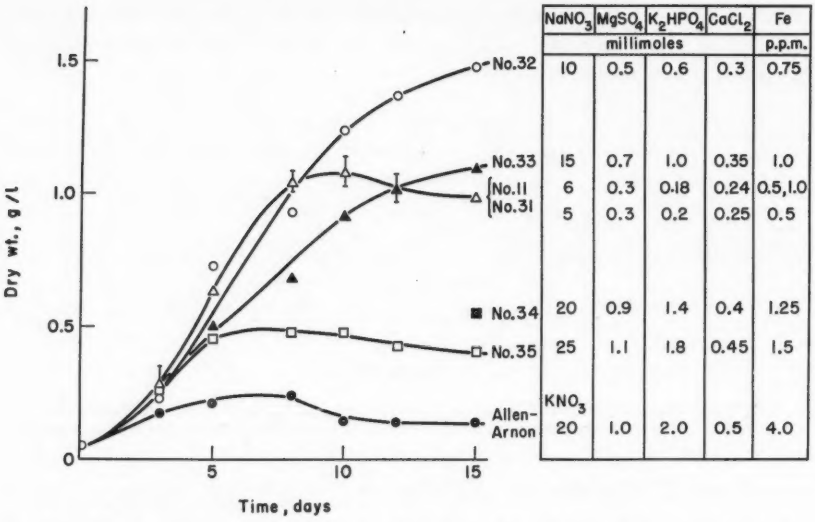



FIG. 4. Changes in pH of 25 ml of medium No. 11 during storage at room temperature in a 50-ml Erlenmeyer flask with cotton stopper from the time of preparation until after autoclaving.

The addition of EDTA to medium No. 11 (Table I) was intended to provide an additional and possibly better chelating agent than citrate at alkaline pH's. Jacobson (24) has shown that at pH 9, 90% of the iron chelated with EDTA in Hoagland's nutrient solution was precipitated in 3 months. Goldberg (18) has reported that 85% of the radioactive iron that was added to sea water at pH 8.0, as either the citrate- or EDTA-complexes was no longer in solution after 6 days. "Versen-ol" (N-hydroxyethylethylenediaminetriacetic acid) and "Versene-diol" (N,N′-dihydroxyethylethylenediaminediacetic acid) are chelates that maintain iron in solution at high pH's (11). Comparative growth tests were therefore made with the usual amount of iron supplied to medium No. 11 as equimolar complexes with citrate, EDTA, "Versen-ol", or "Versene-diol". In short-term experiments, no significant differences in yields were observed between any of the treatments.

*Temperature*

Replicate cultures were inoculated with 4.6 mg/liter of freshly grown alga and incubated at temperatures of 13, 16, 22, 28, 32, 38, and 43° C. The flasks were shaken by hand occasionally. After 2 weeks, growth had occurred only in the cultures incubated at 22° and 28° C. The yields were significantly higher at 28° C than at 22° C. After 6 days, some cultures at 43° C were transferred to 22° C but no growth occurred even after prolonged incubation. The alga was apparently killed at the high temperature. The yields among the cultures that did grow were low, and lysis was observed in many of them. By increasing the temperature from 22 to 27° C the yield (and growth rate) in shake flasks with medium No. 11 was increased from 668 mg/liter in 15 days (Fig. 2) to more than 1000 mg/liter in 7 days (Fig. 5) despite a lower light intensity. With the tower-type fermentors the optimum growth temperature was later found to be higher than 28° C (21).




| $NaNO_3$ | $MgSO_4$ | $K_2HPO_4$ | $CaCl_2$ | Fe |
|---|---|---|---|---|
| millimoles | | | | p.p.m. |
| 10 | 0.5 | 0.6 | 0.3 | 0.75 |
| 15 | 0.7 | 1.0 | 0.35 | 1.0 |
| 6 | 0.3 | 0.18 | 0.24 | 0.5,1.0 |
| 5 | 0.3 | 0.2 | 0.25 | 0.5 |
| 20 | 0.9 | 1.4 | 0.4 | 1.25 |
| 25 | 1.1 | 1.8 | 0.45 | 1.5 |
| $KNO_3$ | | | | |
| 20 | 1.0 | 2.0 | 0.5 | 4.0 |

FIG. 5. Comparative growth in medium No. 11 (or 31) and in media having increased concentrations of the major salts as shown. Results are from single cultures except for media Nos. 11 and 31, which are combined to give averages of triplicates. 50-ml shake flasks, 10 ml of medium, 3000 to 3500 lux, 27° C.

*Other Media*

Medium No. 11 contains a higher total salt concentration than mod. Fitzgerald's medium but is more dilute than other media (Table I) that have given good growth of blue-green algae. Kratz and Myers (27) obtained rapid growth of *Anabaena variabilis*, *Anacystis nidulans*, *Nostoc muscorum*, and *Anabaena cylindrica* in media C and D (Table I). These contain about twice as much nitrate and 3.35 times as much $K_2HPO_4$ as medium No. 11. A medium prepared on the basis of Kratz' and Myers' medium D with 2× $NaNO_3$, 2× $MgSO_4$, but only 1.3× $K_2HPO_4$ as compared with medium No. 11 did not promote better growth of *M. aeruginosa* NRC-1. The maximum yield at 1000 lux and 21° C was 1055 mg/liter in 21 days.

Allen and Arnon (3) report excellent growth of *Anabaena cylindrica* in another medium (Table I) that contains a much higher concentration of minerals than medium No. 11. Five new media, Nos. 31 to 35 (Fig. 5), were prepared by the following procedure: the composition of medium No. 11 was slightly modified to give round figures for the molar concentration of each ingredient. This was called medium No. 31. Media Nos. 32 to 35 had the essential minerals increased step by step to give about the same level in media Nos. 34 and 35 as in the medium of Allen and Arnon. Since a preliminary experiment with medium No. 10 had shown that replacement of two-thirds of the $NaNO_3$ by $KNO_3$ was harmful for *Microcystis*, $NaNO_3$ was used as a nitrogen source, although Allen and Arnon used $KNO_3$. Cultures on medium No. 11 and on Allen and Arnon medium (but with Gaffron's minor

element solution) served as controls. The temperature was increased to 27° C and the amount of medium in each 50-ml flask was reduced to 10 ml to allow more thorough illumination and better aeration of the cultures. The conditions used were a compromise between those used by Allen and Arnon and those used in earlier experiments. Medium No. 32 gave the highest yields (Fig. 5). Further increases in salt concentrations reduced both growth rates and yields. There was little or no growth in Allen and Arnon medium.

Increasing the $NaNO_3$ concentration in medium No. 32 from 10 to 15 or 20 m*M* promoted lysis of the cultures. Growth, which had been similar in all three media for the first 5 days (Fig. 6), was thereby limited.

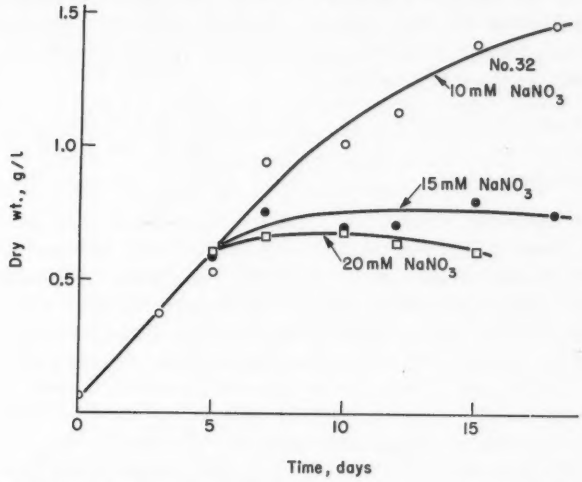



FIG. 6. Growth in medium No. 32 with increasing concentrations of $NaNO_3$. Lysis occurred in the cultures with extra $NaNO_3$ after the 5th day. 50-ml shake flasks, 3000 to 3500 lux, 27° C.

*pH*

Olson (32) reported that the optimum pH for his strain of *Microcystis* was 8. Gerloff, Fitzgerald, and Skoog (16) found an unusually high optimal pH value of approximately 10 for the growth of their strain of *Microcystis*. To raise the pH of the medium, they added sodium carbonate and sodium silicate and sodium hydroxide in one experiment. Experiments with *M. aeruginosa* NRC-1 showed that sodium carbonate and sodium silicate could be omitted without affecting the final yield of the alga. Media Nos. 11 and 32 without added carbonate and silicate had lower initial pH's, which may or may not have been responsible for an initial lag in growth rate that was observed (16). After 5–7 days, however, no significant differences in pH between buffered and unbuffered cultures were observed. After about 15 days, the yields in buffered and unbuffered cultures were equal. Other experiments with slightly buffered medium No. 11 indicated that yields were little affected throughout a pH range of 8 to 11.

In medium No. 11, buffered at pH 8.4 with either 0.01 *M* collidine (2,4,6-trimethylpyridine) or 0.01 *M* Tris (tris(hydroxymethyl)-aminomethane) (19), *M. aeruginosa* NRC-1 did not grow. To promote good growth, the concentration of Tris had to be reduced to less than 0.0016 *M*. At this concentration, however, the buffering effect was negligible.

*Aeration*

The possibility that aeration might be limiting growth in cotton-stoppered flasks was investigated. The amount of gas exchange in such flasks is a function not only of the rate of shaking but also of the surface-to-volume ratio of the medium. This, in turn, is a function of the ratio of flask volume (f.v.) to medium volume (m.v.). The average 25-day yields of triplicate cultures grown in 25 ml of medium No. 11 in 50-ml flasks (f.v./m.v. = 2) with different degrees of shaking were as follows:

| | |
|---|---|
| shaken continuously | 1068 ± 120 mg/liter, |
| shaken once daily | 988 ± 96 mg/liter, |
| unshaken | 832 ± 49 mg/liter. |

Growth was significantly better in shaken as compared with unshaken cultures. There were no significant differences in the yields of cultures that were continuously shaken or were shaken once daily, however. The yields of both continuously shaken and still cultures were found to increase with increasing f.v./m.v. ratio (Fig. 7A). Ratios of 4 or higher plus continuous shaking appeared to be desirable for the achievement of the highest yields.

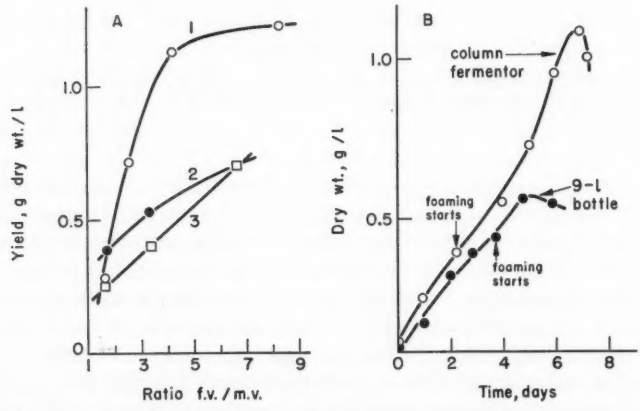



FIG. 7. (A) Effect of the ratio of flask volume: medium volume on yields of single cultures grown for 25 days. Curve 1: 125-ml shake flasks containing 80, 50, 30, or 15 ml of medium, 1000 lux, 19 to 22° C. Curve 2: 1-liter still flasks containing 600, 300, or 150 ml of medium, approx. 2000 lux, 27° C. Curve 3: 250-ml, 500-ml, or 1-liter still flasks containing 150 ml of medium, approx. 2000 lux, 27° C. (B) Comparative growth in medium No. 11 at room temperature in tower-type fermentor and 9-liter bottle. Fermentor: 2.7 liters, 6500 + 4500 lux, 25 to 28° C, 1000 cc/minute aeration. Bottle: 6.25 liters, three "Circline" fluorescents, 25 to 30° C, 4000 cc/minute aeration.

Attempts to improve growth further by bubbling 0.3% $CO_2$ in air at 100 cc/minute through 25 ml of medium in shake flasks proved unsuccessful. With eight of the highest yielding media indicated in Figs. 2 and 3, the extra $CO_2$ slightly reduced rather than increased yields after 26 days. The final pH's ranged from 7.4 to 8.8 as compared with 8.9 to 11.1 for the controls.

*Large-scale Culture: Lysis*

That vigorous aeration (and better illumination) of cultures grown on medium No. 11 in the tower-type fermentor can produce rapid growth was shown in a previous paper (23). The yields obtained were the equivalent of the best obtained in flasks (Fig. 6). Growth at room temperature in the fermentor was compared with growth in a 9-liter-bottle adaptation of the fermentor, using some freshly grown algae from the former to inoculate the latter (Fig. 7B). The initial generation times calculated (30) from the two growth curves were 7 hours for the culture in the 9-liter bottle and 14 hours for the culture in the fermentor. The culture in the bottle had a smaller inoculum. This may have accounted, at least in part, for the higher initial growth rate, since the rates in both cultures declined with increasing cell density. By the fifth day, for example, generation times in the bottle and fermentor had fallen to 80 hours and 70 hours respectively. On the 4th day, both cultures were foaming. This was an indication that severe lysis was taking place. On the 5th day, after a slight increase in growth rate, the culture in the bottle lysed badly and stopped growing when the yield was only 550 mg/liter. The culture in the fermentor grew on without severe lysis and reached a yield of 1100 mg/liter on the 8th day. The pH of both cultures increased from 8.5 to 10.0 during the first 2 days. Thereafter it fluctuated between 9.5 and 9.9 without any noticeable change associated with lysis. In three additional experiments with 9-liter bottles it was found that lysis might occur in varying degree at almost any stage of development. Varying the rate of aeration from 2000 to 6000 cc/minute or using inocula grown under different conditions neither controlled nor prevented lysis.

Still cultures and shake cultures were observed to lyse, too. They stopped growing and changed color from blue-green to milky white—sometimes within a few days. Microscopic examination at the milky green stage showed numbers of pale cells with few pseudovacuoles and "corroded" outlines plus some normal cells. At the milky white stage, only cell fragments were detectable. The phenomenon was first observed in the experiment in which the balance of the three major salts was investigated (Fig. 2). In this experiment, the cultures with the highest salt concentrations gave the highest yields but were the first to lyse.

Lysis could not be correlated with changes in pH, as already mentioned. Increasing the concentration of $Na_2SiO_3$, $Na_2CO_3$, or both from 0.2 to 1.0 m*M* tended to promote lysis. When the concentration of sodium nitrate in medium No. 32 was increased from 10 to 15 or 20 m*M* (Fig. 6) the cultures with extra $NaNO_3$ lysed and ceased further growth. When similar or even higher concentrations of nitrate were supplied as the calcium instead of the

sodium salt there was no lysis (Table III). It appeared to be high concentrations of sodium or a deficiency of calcium that promoted lysis. The higher calcium concentrations caused heavier initial precipitates in the media, which may have been indirectly beneficial. An attempt was made to transmit some lysis-promoting factor or factors from an older, severely lysing culture to a younger, actively growing culture but the results were inconclusive.

TABLE III

The effect of various concentrations of sodium nitrate or calcium nitrate in medium No. 32 upon appearance and lysis; 25-ml shake flasks, 3000 to 3500 lux, 27° C, 14 days

| Nitrogen source | Nitrate conc., m*M* | Color | Lysis |
|---|---|---|---|
| $NaNO_3$ | 10 | Light blue-green | None |
| | 20 | Pale blue-green | Partial |
| | 40 | Milky white | Complete |
| | 80 | Almost water-clear | Complete |
| $Ca(NO_3)_2$ | 10 | Light green | None |
| | 20 | Light green | None |
| | 40 | Light blue-green | None |
| $NaNO_3 + Ca(NO_3)_2$ | 10 + 10 = 20 | Light green | None |

## Discussion

The results of this investigation indicate that the growth rates and yields of *M. aeruginosa* reported by Gerloff *et al.* (16, 17) were far from being the maximum attainable, as, indeed, they were careful to point out might be the case.

Increasing the concentrations of the three major salts in mod. Fitzgerald's medium as much as 4×, plus continuous shaking, produced significantly better yields of *M. aeruginosa* NRC-1 at both 4000 lux and 1000 lux (Figs. 2, 3) than were obtained by Gerloff *et al.* (16, 17). The salt balance in this range of concentrations was not especially critical. Medium No. 11, which contained 4× $NaNO_3$, 3× $K_2HPO_4$, and 3× $MgSO_4$ was adopted as the best combination since the alga retained its blue-green color the longest.

No indications were obtained that the growth of *M. aeruginosa* NRC-1 was limited by a deficiency of minor elements. Chelation with EDTA increased the concentration range of minor elements that was tolerated, but did not improve growth. Soil extract, which was growth-promoting, may have supplied some factor other than the 10 vitamins that were not growth-promoting (Table II). It may have supplied natural chelates to the medium as well but chelation of minor elements, if not of iron (18), by the citrate in the medium was apparently adequate so these probably had little beneficial effect upon growth. Rendering iron more available at high pH's by suitable chelation also failed to improve growth.

By increasing the temperature from 22° C to 27° C and by almost doubling the concentration of major salts in medium No. 11, the highest yield of all was obtained (Fig. 5). Still higher salt concentrations failed to increase

growth rates or yields any further. Allen and Arnon's medium, which produced unusually heavy yields of *Anabaena cylindrica* (3), inhibited the growth of *M. aeruginosa* NRC-1. The inhibition may have been caused by the high total salt concentration, but was more probably caused by an excess of potassium. Sodium is required by some blue-green algae and promotes better growth of others (2, 4, 14), while still others seem to be indifferent as to which alkali ion is the major component of the culture medium (2). Allen (2) has reported that three species of unicellular blue-greens, *Synechococcus cedorum*, *Chroococcus turgidus*, and *Chroococcus* sp., and one *Oscillatoria* sp. not only grow without added potassium, but require sodium for growth in the presence of potassium. Allen and Arnon's medium contains sodium and potassium in the ratio of 0.15. The sodium:potassium ratios for media No. 11 and 32 are 15.0 and 9.2 respectively. Growth was inhibited in medium No. 10 when two-thirds of the $NaNO_3$ was replaced by $KNO_3$ but it was not inhibited by a one-third replacement. The sodium:potassium ratios for these two media are 0.67 and 1.99, respectively. This indicates that *M. aeruginosa* NRC-1 requires a ratio of 1 or higher to prevent growth inhibition. The requirements and tolerances for potassium and sodium in relation to the optimal ratio of the two elements need to be carefully established for *M. aeruginosa* NRC-1 and also for other species of blue-green algae.

*M. aeruginosa* NRC-1 gave equally good yields in both buffered and unbuffered medium No. 11 over a pH range of 8 to almost 11. The pH optimum for this species is evidently not so pronounced as previous work (16, 32) has indicated. When the medium was buffered at pH 8.4 with either collidine or Tris there was no growth. Tris is used to buffer media for algae and bacteria but toxic effects have been noted with both (25, 26, 28, 33, 34). With a marine blue-green alga, *Phormidium persicinum*, Tris is a suitable buffer as long as its antagonism of potassium utilization is overcome by the addition of more potassium to the medium (33).

An examination of the growth curves for cultures in shake flasks, the tower fermentor, or the 9-liter bottle (Figs. 5, 6, and 7B) reveals the lack of a distinct period of exponential growth in all three. A calculation of generation times (30) showed that they were shortest for the first day, and became longer for each day thereafter until growth stopped in 5 to 15 or more days, depending upon culture conditions. The initial generation times were 7, 14, and 28 hours for cultures in the 9-liter bottle, the fermentor, and the shake flasks, respectively. Leakage and/or lysis could be detected in the highly aerated cultures by foaming on the 3rd or 4th days (Fig. 7B). Leakage and/or lysis could also be detected prior to foaming by the presence of the fast-death factor in culture filtrates (23). The early decline in growth rate suggests that leakage and/or lysis may be occurring continually and that some of the products released into the medium act as growth inhibitors. In support of this is the fact that cells from a culture that foamed badly and had ceased to grow were no longer viable (23). An analysis of the filtrate taken from a foaming culture revealed the presence of rather large amounts of polysaccharide and polypeptide materials. Other blue-green algae, notably *Anabaena cylindrica*, are known

to release such compounds into the culture medium (7, 13) and Fogg (14) has suggested that extracellular polypeptides have an important effect on growth of the algae concerned. It is possible that vigorous aeration and agitation may promote rapid growth and high yields (Figs. 7A, 7B) not only by insuring better gas exchange and more efficient utilization of light but also by helping to relieve inhibition caused by substances released into the medium.

The shortest generation time on record for a blue-green alga is 1.5 to 1.7 hours calculated from the data of Kratz and Myers (27) for *Anacystis nidulans* grown for short periods at 41° C. This alga grew exponentially for periods up to 24 hours and gave yields of approximately 330 mg dry wt./liter in these times. Allen and Arnon (3) reported extraordinary yields for *Anabaena cylindrica* of 7 to 8 g dry wt./liter in 5 to 6 days. The shortest initial generation time calculated from their data was 24 hours. Significant, perhaps, in connection with the foregoing discussion of inhibitors, is the fact that the growth rate of *Anabaena cylindrica* (3) also declined steadily after the first day like that of *M. aeruginosa* NRC-1.

The lysis of blue-green algae does not seem to have been observed or commented on before. The mineral composition of the medium seems to have some influence upon its severity, if not upon its occurrence (Figs. 2, 6). An excess of sodium (or deficiency of calcium) seems to promote it (Table III). However, in other experiments under constant conditions, severe lysis appeared to occur at random. This suggests the involvement of other, as yet unknown, factors. The bacterial contaminants are naturally suspect, but the details of their role, if any, have still to be thoroughly investigated. It is well known that waterblooms of blue-green algae sometimes disappear very rapidly. Lysis may well account, at least in part, for this rapid disappearance. Aleev (1) has examined the autolysis of two species of green algae and concluded that it plays an important part in the accumulation of organic substances in the surroundings inhabited by algae.

The growth rates and yields of *M. aeruginosa* NRC-1 reported in this and in a previous paper (23) are the highest yet achieved for this species in laboratory culture. The cell densities that have been observed exceed those that have been reported for heavy waterblooms (6, 31, 36). Further detailed study of mineral nutrition, culture conditions, and lysis may result in the achievement of still higher growth rates sustained over longer periods with even greater yields. Meanwhile, the method of culture of *M. aeruginosa* NRC-1 in 9-liter bottles developed in this study has been successfully adapted (8, 20) to the production of kilogram quantities of dried algae for the isolation and identification of the fast-death factor and for toxicity tests on large animals.

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# SOME SEROLOGICAL PROPERTIES OF SUBSTANCES EXTRACTED FROM STAPHYLOCOCCI AND A HEMOLYTIC STREPTOCOCCUS[1]

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## Abstract

Polysaccharide-type extracts prepared from a coagulase-positive staphylococcus, a coagulase-negative staphylococcus, and a type 12 hemolytic streptococcus were investigated by means of the hemagglutination technique to determine whether the extract of *Staphylococcus pyogenes* contained antigens that could detect species-specific antibodies in antisera. The sera used were prepared by inoculating rabbits with vaccines, extract-sensitized erythrocytes, or extract alone. The extracts themselves elicited poor antibody formation unless adsorbed on erythrocytes. Marked serological cross reactions were found among the preparations from all three organisms, and mirror absorption tests failed to yield clear evidence of species-specific factors in the extracts or in the antisera. The latex fixation technique demonstrated similar cross reactions to those found by hemagglutination. The common antigen or complex of antigens found in the extracts of all three organisms were not identified as the non-specific antigens described by Rantz and others. Chemical analyses of the extracts showed that, as prepared, they were complex mixtures of substances to which the term "polysaccharide" could not properly be applied.

## Introduction

The literature contains little information about antibodies to the somatic antigens of staphylococci, and even less about the part they may play in infections. The few reports which have been published differ on the antigenic specificity of these extractable somatic antigens.

Rountree and Barbour (7) obtained promising results using a hemagglutination test with erythrocytes sensitized with an extract of coagulase-positive staphylococci prepared by a modification of the method of Warburton *et al.* (10). Similar findings were reported by Oeding (3) with various staphylococcal polysaccharide products. Extracts prepared by Pakula and Walczak (5) and by Rantz *et al.* (6), however, lacked species specificity for staphylococci.

Preliminary studies of extracts prepared from coagulase-positive staphylococci, coagulase-negative staphylococci, and a hemolytic streptococcus showed a marked degree of serological cross reaction. The common properties of these extracted substances were investigated and are reported here.

## Materials and Methods

### *Strains*

Three strains of microorganisms were used: a coagulase-positive staphylococcus (SP), a coagulase-negative staphylococcus (SN), and a hemolytic

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streptococcus (ST). SP was isolated from the nares of a healthy carrier. It was of phage type 81 and was resistant to penicillin and the tetracyclines. SN was also recovered from the nose of a human. Streptococcus ST was of Lancefield group A and Griffith type 12.

*Preparation of Extracts—"Polysaccharide"*

The method was based on that described by Warburton *et al.* (10) for the extraction of polysaccharide from *Hemophilus influenzae.* Fresh cultures of each bacterium were inoculated into 125-ml flasks containing a layer of 30 ml of trypticase soy agar (B.B.L.) under 10 ml of brain heart infusion broth (Difco) (9). The flasks were agitated gently on a mechanical shaker for 18 hours at 37 degrees C. For streptococci, 10% horse serum was added to the broth and 5% sheep's blood to the agar.

The bacteria from each 120 ml of broth culture were collected by centrifugation, mixed with 40 ml of 90% phenol, and heated for 10 minutes in a boiling water bath. After centrifugation, the sediment was washed once with 40 ml of 90% phenol, once with the same volume of 95% ethyl alcohol, and twice with ether. To each sediment was added 10 ml of distilled water and the suspensions from a number of batches were pooled and placed in a boiling water bath for 10 minutes. This extraction was continued at room temperature until the next day when the suspension was centrifuged and filtered through a sintered glass filter. The filtrate was added to three volumes of 95% ethyl alcohol to which a few drops of saturated sodium acetate had been added. The precipitate formed at room temperature was collected by centrifugation. The original sediment prior to filtration was re-extracted in water and the process repeated until no further precipitation occurred when the watery supernatant was mixed with alcohol.

The precipitates from each extraction were washed once with alcohol and twice with ether, and finally the pooled yields were re-extracted in water, refiltered, reprecipitated in alcohol and sodium acetate, and washed in alcohol and ether.

The final yield was stored in a desiccator at 4 degrees C. Stock solutions were prepared by dissolving weighed amounts in physiological saline. Such solutions, when refrigerated, were found to be stable for at least 10 weeks.

*Antisera*

Three types of antisera were prepared for each microorganism by courses of intravenous injections into rabbits. The three antigens used were:

(1) Vaccines killed by alcohol as used by Hobbs (2).*
(2) Saline solutions of extract in dosages comparable to those used by Hayes (1).
(3) Human group O erythrocytes sensitized with extract (Oeding (3)).

*Antisera were not prepared with streptococcal vaccines. Grouping sera for Lancefield group A were used instead. These were kindly supplied by Dr. E. T. Bynoe of the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, for the early work, and final experiments were done using similar antistreptococcal grouping sera purchased from Difco Laboratories, Inc.

The sera of the rabbits before immunization did not agglutinate either intact bacterial cells or specifically sensitized erythrocytes. The final antisera did not agglutinate unsensitized guinea pig erythrocytes. All antisera were stored at −20 degrees C.

*Agglutination Tests*

Hobbs' (2) technique was followed. Bacterial suspensions previously heated to 100 degrees C and rapidly cooled were mixed with serial dilutions of serum on a glass slide. These were rotated gently and examined for macroscopic agglutination within 15 minutes.

*Hemagglutination Tests*

Fresh, thrice-washed guinea pig erythrocytes were sensitized by mixing 1 ml of extract solution in saline (1 mg/ml) and 0.1 ml of packed cells. After 1 hour at 37 degrees C the cells were washed twice in saline and diluted to a 1% suspension.

Equal volumes (0.3 ml) of the suspension of sensitized cells and of serial twofold dilutions of serum were mixed in 10×75 mm test tubes and incubated for 1 hour at room temperature with occasional shaking. Each tube was centrifuged for 1 minute at 1000 r.p.m. before reading. The titers were recorded as the reciprocal of the highest dilution showing macroscopic agglutination.

*Latex Agglutination*

The method used was essentially that described by Singer and Plotz (8). From the stock suspension of polysterene latex* a standard suspension was prepared which when diluted 1:100 for use, gave a direct reading of 530 units in a Klett-Summerson photoelectric colorimeter with a red filter at 660 millimicrons. The diluent was borate buffer at a pH of 8.2. This buffer was used in all tests.

An exact determination of the optimal dose of each extract for latex sensitization was necessary because it was not practical to wash the particles by centrifugation at ordinary speeds of 3000 r.p.m. or less. Excess amounts of extract used to sensitize the latex, as well as inadequate quantities, failed to give the maximal titers of antisera. The optimal dose required to sensitize 10 ml of diluted latex varied considerably from extract to extract. It was 0.04 mg of the dried extract of the coagulase-positive staphylococcus, 2.0 mg of that from the coagulase-negative staphylococcus, and 0.8 mg of the streptococcal extract.

The latex was sensitized immediately before use by mixing a predetermined optimal amount of each extract with a standard amount of diluted suspension of latex and allowing the mixture to incubate for 1 hour at room temperature. The titers of antisera were found to be the same whether the sensitized latex was used almost immediately after mixing or kept at room temperature for 4 to 6 hours.

*The stock suspensions of latex were kindly supplied by the Dow Chemical Company, Midland, Michigan, through Dr. J. W. Vanderhoff, and from Dr. R. W. Lamont-Havers, Medical Director, the Arthritis and Rheumatism Foundation, New York. The average diameter of the latex particles was stated to be 0.82 microns.

Antisera were titrated by mixing 0.5 ml of serial twofold dilutions of serum in borate buffer with 0.5 ml of the sensitized latex suspension and incubating in a water bath at 56 degrees C for 4 hours. Incubation at lower temperatures or for shorter periods of time was found unsatisfactory. Readings of the degree of agglutination were taken after centrifuging the tubes at 2300 r.p.m. for 3 minutes.

*Absorption of Sera*

The antisera were absorbed with either (1) packed organisms which had been heated to 100 degrees C and cooled, or (2) guinea pig erythrocytes sensitized with suitable polysaccharide. The absorbing agent was mixed with 4 ml of a serum dilution of 1:5. This was held for 1 hour at 37 degrees C when whole organisms were used, and at room temperature when absorbing was done with sensitized erythrocytes. The mixtures were kept in the refrigerator overnight, and absorptions were repeated if homologous antibody was still present. Control sera were warmed and cooled for the same lengths of time, and where the test sera were absorbed with sensitized erythrocytes, the control sera were treated with unsensitized erythrocytes.

## Results

*I. Antibody-stimulating Properties of Whole Bacteria, Bacterial Extract, and Extract-sensitized Erythrocytes*

*(1) Antisera Produced by Whole Bacteria*

Sera taken at different times and from different rabbits inoculated with a vaccine of the coagulase-positive staphylococcus (SP) were titrated by the hemagglutination technique and by direct bacterial agglutination using homologous reagents. The hemagglutinin titers varied in different samples from 160 to 640, while bacterial agglutination titers ranged from 160 to 320.

Antisera prepared with a vaccine of the coagulase-negative staphylococcus (SN) showed hemagglutinin titers within the same range when tested with erythrocytes sensitized with homologous extracts. Bacterial agglutination titers could not be determined satisfactorily because the strain was agglutinated spontaneously by control serum.

The hemagglutination titers of different antistreptococcal sera, estimated with erythrocytes sensitized with extracts of type 12 streptococci varied from 40 to 640. Bacterial agglutination titers could not be determined because of spontaneous clumping.

*(2) Antisera Produced by Extract Alone*

The extracts themselves were poorly antigenic when injected into rabbits.

The SP extract stimulated antibodies to a maximum titer of 160 as determined by hemagglutination with homologous extract, and the serum agglutinated a suspension of SP cells in a dilution of only 20.

A similarly low hemagglutinin titer, 80 to 160, was found in the antisera produced by the extract of SN.

Streptococcal extracts injected into rabbits failed to stimulate detectable hemagglutinins.

*(3) Antisera Produced by Erythrocytes Sensitized with Extract*

Unlike those given extract alone, rabbits inoculated with extract fixed to human erythrocytes produced high antibody levels. Titrated with homologous materials, the antisera produced by extracts of SP had hemagglutinin titers of 320 to 640 and they agglutinated suspensions of staphylococcus SP in dilutions of 160 to 320. The hemagglutinin titers of antisera against SN and ST were from 320 to 640.

*II. Serological Cross Reactions*

When the hemagglutinin titer of each antiserum was determined using heterologous extracts to sensitize the erythrocytes, marked cross reactions were apparent among all three microorganisms. This crossing was as evident in antisera prepared with vaccines as in those stimulated by injections of sensitized erythrocytes. Several batches of serum were used and all gave similar results. There was such lack of specificity that it was impossible to differentiate between the various extracts and the various antisera by the titers obtained.

Representative results of these hemagglutinin titrations are shown in Table I.

TABLE I

Hemagglutinin titers of unabsorbed sera produced by vaccines, extracts, and extract-sensitized erythrocytes

| | Extract used to sensitize test erythrocytes | | |
|---|---|---|---|
| | SP | SN | ST |
| Serum type* | Hemagglutinin titer† | | |
| Vaccine serum | | | |
| SP | 160 | 80 | 80 |
| SN | 160 | 160 | 80 |
| ST | 160 | 160 | 160 |
| Extract serum | | | |
| SP | 160 | 160 | 160 |
| SN | 80 | 80 | 80 |
| ST | 10 | 10 | 10 |
| Extract-cell serum | | | |
| SP | 640 | 320 | 640 |
| SN | 640 | 320 | 640 |
| ST | 640 | 640 | 640 |

*Vaccine serum prepared by inoculating whole organisms. Extract serum prepared by inoculating extract alone. Extract-cell serum prepared by inoculating extract-sensitized erythrocytes.

†Titer recorded as reciprocal of dilution. SP = coagulase-positive staphylococcus; SN = coagulase-negative staphylococcus; ST = streptococcus.

Cross reactions were also found when titers were determined by bacterial agglutination as shown in Table II. The crossing was more marked in those antisera produced by extract-sensitized erythrocytes than in those prepared with vaccines. As previously noted, bacterial agglutination could not be done with suspensions of SN or ST because of spontaneous agglutination.

TABLE II

Direct agglutination titers of unabsorbed sera produced by vaccines and extract-sensitized erythrocytes

| | Serum and type | | | | | |
|---|---|---|---|---|---|---|
| | Vaccine serum | | | Extract-cell serum | | |
| | SP | SN | ST | SP | SN | ST |
| | Agglutinin titer | | | | | |
| Suspension of SP | 320 | 80 | 0 | 160 | 160 | 80 |

*III. Absorption Tests*

Mirror absorption tests were done with those reagents which might have been expected to be most homogeneous and to have the least complexity of factors, namely, antisera prepared by inoculating rabbits with extracts fixed to erythrocytes and using extract-sensitized erythrocytes both for absorptions and for titrating residual antibodies. Representative findings are given in Table III.

Absorption was complete (titer of less than 10) in all but one instance when antisera were absorbed with homologous antigens. The single exception was a residual titer of 10 to SP extract found in the antistreptococcal serum after absorption with streptococcal extract.

Following absorption of sera with heterologous extracts, there was a marked reduction in the titers of all antisera against the other extracts. This reduction was often complete and 64-fold in extent, e.g., 640 down to 0. It was never less than eightfold, e.g., 320 down to 40. Residual titers did not exceed 40 in the nine instances where they were found, and it is noteworthy that the streptococcal extract was the absorbing agent in seven of the nine such cases.

TABLE III

Hemagglutinin titers after absorption of antisera to sensitized cells with sensitized erythrocytes

| | | Extract used to sensitize cells | | |
|---|---|---|---|---|
| | | SP | SN | ST |
| Antiserum | Absorbing agent | Hemagglutinin titer | | |
| SP | None | 640 | 320 | 640 |
| | SN | 10 | 0 | 0 |
| | ST | 40 | 20 | 20 |
| | SP | 0 | 0 | 0 |
| SN | None | 640 | 320 | 640 |
| | SP | 0 | 20 | 0 |
| | ST | 20 | 40 | 20 |
| | SN | 0 | 0 | 0 |
| ST | None | 640 | 640 | 640 |
| | SP | 0 | 0 | 0 |
| | SN | 0 | 0 | 0 |
| | ST | 10 | 0 | 0 |

Whatever interpretation one might give to these low residual titers following absorptions, it seems obvious that there is a major antigen, or complex of antigens, involved in these reactions. Furthermore, this antigen, the predominant one in extracts of all three organisms, lacks species specificity.

The low residual titers found after absorptions are difficult to explain. Two interpretations were considered.

The simpler is that they are not significant and are related to the vagaries of absorption and hemagglutination tests generally or to technical errors. Similar results were found repeatedly in other tests. The alternative explanation is that they are due to still other antigens of a more or less specific nature which are present in small quantities, and which stimulate corresponding antibodies to low or irregular concentrations.

All the findings in Table III could be explained by postulating the existence of three, or perhaps four, antigens. Antigen "*a*" is the major, non-specific or common one discussed above. It is present in relatively large quantities in all three extracts as are antibodies against it in all three antisera. Minor antigen "*b*" is specific for the coagulase-positive staphylococcus, and minor antigen "*c*" is found only in the extract of the coagulase-negative staphylococcus. Minor antigen "*d*" is common to all and may only be a part of the "*a*" complex. One must assume, however, that antigen "*d*" differs somewhat in its properties at different times and depending on the type of cell from which it is extracted. Such a postulate is not without precedent. Oeding (3), for example, noted a difference in the activity of certain antigens in different staphylococcal extracts.

Irrespective of its source antigen "*d*" seems capable of sensitizing erythrocytes effectively both for stimulating antibody formation and for titrating hemagglutinins. When derived from staphylococci, it will absorb antibody readily from all antisera. In streptococcal extracts, however, it absorbs antibody poorly from antisera prepared with heterologous organisms even though it will absorb antibody readily from antistreptococcal sera. The notably poor absorptions were of staphylococcal antisera with streptococcal extracts. The antigen in these extracts may be primarily a streptococcal one which is present in staphylococci in a related but not identical form.

Reciprocal absorption tests were also done with combinations of reagents which might be called non-homogeneous as to the methods used for preparation of sera, absorption, or titration. Table IV shows the hemagglutinin titers when antisera prepared with sensitized erythrocytes were absorbed with vaccines. Table V gives the results when antisera produced with vaccines were absorbed with sensitized erythrocytes, and Table VI, when antisera produced with vaccines were absorbed with vaccines. The antibodies detectable by hemagglutination were limited of course in all cases to those corresponding to antigens present in extracts or more precisely to those capable of sensitizing erythrocytes.

Again the main finding is similar to that in Table III. There is a marked reduction in all titers following all absorptions. This indicates again the presence of a major antigen common to all three microorganisms.

There were low residual titers after absorption in 28 instances and as before, this occurred most frequently after absorptions with streptococcal antigens (20 of 28 tests). The highest residual titers were also found after absorptions with streptococci or their products. Again, the streptococcal reagents removed antibodies active against streptococci most successfully from antistreptococcal sera but poorly from antistaphylococcal sera.

The evidence from these non-homogeneous absorptions for the specific antigens "*b*" and "*c*" postulated earlier is less clear. This might be expected because of the complexities introduced by the use of so-called non-homogeneous reagents. Nevertheless, the results in Table IV and VI suggest the presence

TABLE IV

Hemagglutinin titers after absorption of antisera to sensitized cells with vaccines

| Antiserum | Absorbing agent | Extract used to sensitize cells | | |
|---|---|---|---|---|
| | | SP | SN | ST |
| | | Hemagglutinin titer | | |
| SP | None | 320 | 640 | 320 |
| | SN | 10 | 0 | 10 |
| | ST | 80 | 40 | 10 |
| | SP | 10 | 0 | 0 |
| SN | None | 320 | 320 | 640 |
| | SP | 0 | 0 | 0 |
| | ST | 40 | 40 | 10 |
| | SN | 10 | 0 | 0 |
| ST | None | 320 | 320 | 320 |
| | SP | 0 | 0 | 0 |
| | SN | 0 | 0 | 0 |
| | ST | 20 | 20 | 0 |

TABLE V

Hemagglutinin titers after absorption of antivaccine sera with sensitized erythrocytes

| Antiserum | Absorbing agent | Extract used to sensitize cells | | |
|---|---|---|---|---|
| | | SP | SN | ST |
| | | Hemagglutinin titer | | |
| SP | None | 160 | 80 | 80 |
| | SN | 0 | 0 | 0 |
| | ST | 20 | 40 | 10 |
| | SP | 0 | 20 | 0 |
| SN | None | 160 | 160 | 80 |
| | SP | 0 | 20 | 0 |
| | ST | 20 | 40 | 10 |
| | SN | 0 | 0 | 0 |
| ST | None | 160 | 320 | 160 |
| | SP | 0 | 10 | 0 |
| | SN | 0 | 0 | 0 |
| | ST | 20 | 10 | 0 |

TABLE VI

Hemagglutinin titers after absorption of antivaccine sera with vaccines

| Antiserum | Absorbing agent | Extract used to sensitize cells: SP | SN | ST |
|---|---|---|---|---|
| | | Hemagglutinin titer | | |
| SP | None | 160 | 320 | 80 |
| | SN | 10 | 0 | 0 |
| | ST | 0 | 20 | 0 |
| | SP | 0 | 0 | 0 |
| SN | None | 160 | 160 | 320 |
| | SP | 0 | 0 | 0 |
| | ST | 10 | 80 | 0 |
| | SN | 0 | 0 | 0 |
| ST | None | 160 | 160 | 160 |
| | SP | 0 | 0 | 0 |
| | SN | 0 | 0 | 0 |
| | ST | 0 | 10 | 0 |

of antigen "*b*", specific for the coagulase-positive staphylococcus. There is no further support for specific antigen "*c*" in extracts of the coagulase-negative staphylococcus. Evidence for a specific streptococcal antigen is lacking. It is impossible to assess the significance of the low residual titers after absorptions. Absorptions of sera prepared against polysaccharide alone were not done as these sera were of low titer and it was thought little more information would be obtained.

*IV. Latex Agglutination Tests*

Various unabsorbed antisera were titrated with latex particles sensitized by extracts. It was hoped that latex particles might adsorb different constituents of the extracts than did erythrocytes and that these might give more specific reactions than those found by hemagglutination.

The results of these tests are given in Table VII. They are similar generally to those found by hemagglutination and show approximately the same degrees of cross reaction. Absorption tests were not done.

*V. Chemical Composition of Extracts**

Attempts were made to determine the chemical nature of the extracts. These confirmed our belief that they were complex mixtures of substances. This was further substantiated by the multiple lines of precipitation found when the extracts and antisera were examined serologically by the agar diffusion method of Ouchterlony (4).

The dried extract from SP was found to contain 9.7% nitrogen and 12.8% phosphorus of which 11% was organic phosphorus. The extract gave strongly positive ninhydrin and Sakaguchi reactions indicating the presence of a considerable amount of protein which was estimated to account for 11% of the

*We are indebted to Dr. J. M. Neelin, at present of the National Research Council, Ottawa, who did the chemical determinations for us.

TABLE VII

Latex agglutination and hemagglutination. Comparative titers and cross reactions with unabsorbed antisera

| | Extract used to sensitize | | | | | |
|---|---|---|---|---|---|---|
| | SP | | SN | | ST | |
| | Latex | Hemagg. | Latex | Hemagg. | Latex | Hemagg. |
| Serum type* | Titers | | | | | |
| Vaccine serum | | | | | | |
| SP | 640 | 1280 | 160 | 320 | 40 | 160 |
| SN | 20 | 320 | 320 | 320 | 20 | 320 |
| ST | 160 | 40 | 20 | 40 | 40 | 40 |
| Extract-cell serum | | | | | | |
| SP | 640 | 640 | 80 | 640 | 80 | 640 |
| SN | 320 | 640 | 320 | 640 | 160 | 640 |
| ST | 320 | 640 | 160 | 640 | 160 | 640 |
| Extract serum | | | | | | |
| SP | 40 | 160 | 80 | 160 | 20 | 160 |
| SN | 40 | 80 | 160 | 80 | 20 | 80 |
| ST | 20 | 10 | 40 | 10 | 0 | 10 |

*Vaccine serum prepared by inoculating whole organisms. Extract-cell serum prepared by inoculating extract-sensitized erythrocytes. Extract serum prepared by inoculating extract only.

total dry weight. The ultraviolet absorption spectrum was characteristic of nucleotides or degraded nucleic acid. Nearly half of the organic phosphorus was estimated to be the phosphate of the degraded nucleic acid; this nucleic acid would constitute about 47% of the total dry weight of the sample. If the remaining organic phosphorus was in the form of phosphate, it would amount to 21% of the total dry weight.

The sample from strain SN contained slightly more protein (15%) but the least nucleotide material (24%), while that from the streptococcus contained the least protein (5%) but only slightly less nucleotide (43%) than that of SP.

When one considers the chemical complexity of these extracts and the possible interplay of their constituents in serological tests because of such variable factors as antigenicity and adsorption to erythrocytes, the difficulty of interpreting the results of serological absorption tests is readily understood. It is clear also that the term "polysaccharide" is a misnomer when applied to these extracts.

## Discussion

The original purpose of this work was to develop a method of detecting and titrating antibodies specific for staphylococci in the sera of animals. The antibodies sought were those directed against cellular constituents rather than those against such products as exotoxins. The hemagglutination technique seemed appropriate because it allowed the use of chemical extracts of cells. Since so-called "polysaccharide" antigens of other organisms have been widely used, extracts of this type seemed to offer the greatest promise.

The aim was not achieved. While the hemagglutination technique detected antibodies in high titers in various prepared antisera, they lacked specificity for the coagulase-positive staphylococcus used. They could not be differentiated from antibodies stimulated by coagulase-negative staphylococci or hemolytic streptococci. Some useful findings emerged nevertheless.

The observation, first made by Rountree and Barbour (7) and later by others, that antibodies against polysaccharide extracts of staphylococci can be detected by the hemagglutination technique has been confirmed. That cross reactions occur between extracts from staphylococci and from streptococci, as reported by Rantz *et al.* (6), has also been shown. These cross reactions, although not proved to be due to the same common antigen described by Rantz, were of major degree and occurred between coagulase-positive staphylococci, coagulase-negative staphylococci, and hemolytic streptococci.

The "polysaccharide" extracts by themselves lacked the ability to stimulate good antibody formation in rabbits. When adsorbed on erythrocytes, however, they became more fully antigenic.

The latex fixation technique can be adapted to detect antibodies against certain components in extracts of staphylococci and streptococci.

Finally, the work indicates that the so-called "polysaccharide" extracts prepared from these microorganisms by the methods used are far less homogeneous, chemically and serologically, than is often believed. Only one major common antigen, or complex of antigens, was found. There were minor variations and discrepancies suggesting that various antigenic substances were present, with some showing qualitative as well as quantitative differences in different extracts. The streptococcal extract, for example, seemed similar antigenically to the others but with poorer power of absorbing antibody. The complexity of the extracts was also evident from chemical analysis. There were suggestions that some species-specific antigens were present, even though in small amounts. If this is so, it should be possible to isolate them by other methods of extraction and purification.

Wiley (11) has recently described a mucoid strain of coagulase-positive staphylococcus which, together with certain other strains, undergoes capsular swelling in the presence of immune serum. Suitable extracts from this or similar strains might provide antigens with greater specificity for pathogenic staphylococci than those studied here. Furthermore, the hemagglutination technique with such extracts could become a valuable tool in studying the immunology of staphylococcal disease, especially if the presence of this capsular material were proved to correlate with virulence. Further investigations along these lines are required and will present a challenge to the chemist as well as the immunologist.

We are grateful to Professor P. H. Greey, Department of Bacteriology, University of Toronto, for suggestions in the preparation of this manuscript.

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## NOTES

### THE EFFECT OF ROSE BENGAL ON POLYSACCHARIDE PRODUCTION IN SOILS

D. L. LYNCH[1]

Polysaccharide production in soils as affected by organic amendments and other factors has been studied by Rennie *et al.* (2) and by Chesters *et al.* (1). In these studies the highest levels of polysaccharide production were noted 7–10 days after these materials were incorporated into soils. Polysaccharide production was also found to be correlated with the aggregate stability of these soils.

Rose bengal, a bacteriostatic dye, was utilized to delineate and evaluate polysaccharide production further from the standpoint of the kind of population responsible for this production. The experiments were carried out on a Pocomoke sandy loam (on both forest-covered and cultivated soil) with a pH of 6.5. This is a very nearly optimal pH for crop growth. Two per cent additions by weight of sucrose, pectin, and casein were added to the soils, except for the controls. The rose bengal was added to one set of the soils replicates at the rate of 1:15,000 in a water solution. The population in the rose bengal amended soil was thus maintained by bringing the soil moisture level up to an optimum with the rose bengal solution every few days rather than with water alone as in the case of the bacterial populations.

While the presence of the dye appeared to have lessened the activities of the bacteria, in terms of polysaccharide production, plate counts also indicated some degree of success by the dye in lowering the number of bacteria at this soil pH, but the populations under both conditions were fairly heterogenous. Qualitative changes undoubtedly had taken place since changes were observed in kinds and numbers of bacteria between the amended and unamended soils. A slight increase in bacteria that would grow on rose bengal agar also was evident in the amended soil. The polysaccharides were determined by precipitation with acetone from the fulvic acid fraction according to the method of Rennie *et al.* (2).

The efficiency of polysaccharide production by microorganisms in a rose bengal amended and unamended soil is given in Table I. After 2 weeks of incubation the population in an unamended soil in every case had produced more polysaccharides than had a microbial population in soil amended with rose bengal. The greatest differences in the amounts of polysaccharides produced by the two populations resulted from the addition of sucrose.

Both populations on pectin-amended soils produced greater amounts of soil polysaccharides than on soils amended with sucrose and casein. Relatively small amounts of polysaccharides were produced on a casein-amended

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soil probably because of the narrow C:N ratio of this protein. As can be seen in Table I the average yields of polysaccharides were much greater with a population grown in the absence of rose bengal than when rose bengal was present. However, there were no appreciable differences between the same populations on the cultivated or forested soils. It seems apparent from this that a microbial population in the absence of rose bengal produces a higher yield of polysaccharide than one grown in the presence of this bacteriostatic dye. Further work is needed to determine if the same parallel exists in regard to soil aggregation.

TABLE I

The production of microbial gums as affected by addition of sucrose, pectin, and casein on two soils after 2 weeks of incubation[a]

| | Treatment | | | |
|---|---|---|---|---|
| | No rose bengal | | Rose bengal added | |
| | Gums as lb/ac | | | |
| | Field soil[b] | Forest soil[c] | Field soil[b] | Forest soil[c] |
| Control | 480 | 1120 | 460 | 910 |
| Sucrose | 4970* | 4220* | 1980 | 1650 |
| Pectin | 5700* | 4980* | 3740* | 3750* |
| Casein | 2610* | 2630* | 1650 | 1530* |
| Av. | 3438 | 3438 | 1957 | 1960 |

[a]Those results which are significant are indicated by *.
[b]L.S.D. for the treatment with the field soil at 1% was ± 1740.
[c]L.S.D. for the treatment with the forest soil at 1% was ± 1321.

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## SOME ASPECTS OF PHOSPHATE METABOLISM OF CLAVICEPS PURPUREA (FR.) TUL.[1]

C. DE WAART[2] AND W. A. TABER

The production of secondary metabolic products, such as antibiotics by fungi, eubacteria, and actinomycetes, is commonly associated with slow growth and with low concentrations of orthophosphate in the medium (1, 2, 4, 5, 9). Similar conditions of slow growth, inefficient utilization of carbon for growth (11, 12), and a low concentration of orthophosphate are also required for production of the ergot alkaloids by *Claviceps purpurea*. The quantity of phosphate which permits alkaloid synthesis is insufficient for maximum rate of growth (Figs. 1 and 2). These data led to the hypothesis that a high concentration of phosphate in the medium favored abundant synthesis of cells and phosphorylated compounds, and that the ergot alkaloids were formed when there was not enough phosphate for the operation of these "normal" activities.

Data already exist in the literature which attest to the fact that a high concentration of orthophosphate is associated with abundant cellular and nucleic acid synthesis but not with antibiotic synthesis (2, 4). High yields of polyphosphates, on the other hand, has been related to restricted growth (6) or to media which may not be optimal for growth (7).

The course of formation of nucleic acids and condensed phosphate compounds under conditions which both favor and prevent the synthesis of ergot alkaloids has now been studied in this laboratory in order to ascertain whether or not the synthesis of shunt metabolism products is correlated with some particular mode of phosphate metabolism. The preliminary studies on stationary cultures grown in a low concentration of orthophosphate revealed that even under this condition of partial phosphate starvation both nucleic acids and polyphosphates were formed. Moreover, after the disappearance of inorganic phosphate from the medium, there was a continuous release of nucleic acids and polyphosphates from the mycelium, without any apparent curtailment of growth. Although the phenomenon of nucleic acid leakage during growth of bacteria is well known, the loss of nucleic acids and polyphosphates by fungi during growth has not been reported as yet to the authors' knowledge.

Mycelium and filtrate of cultures of *Claviceps purpurea*, strain PRL 1578, grown for various periods of time, were separated for analysis. The cold 5% trichloroacetic acid (TCA) extraction procedure (8, 13) was used for isolating polyphosphates and low-polymerized nucleic acids. Phosphate content was estimated as free inorganic phosphate in filtrate and extract (10) and as total phosphate in extracted mycelium, filtrate, and extract, respectively, by digesting the sample with sulphuric acid (3) before carrying out the phosphate

[1]Issued as N.R.C. No. 6029.
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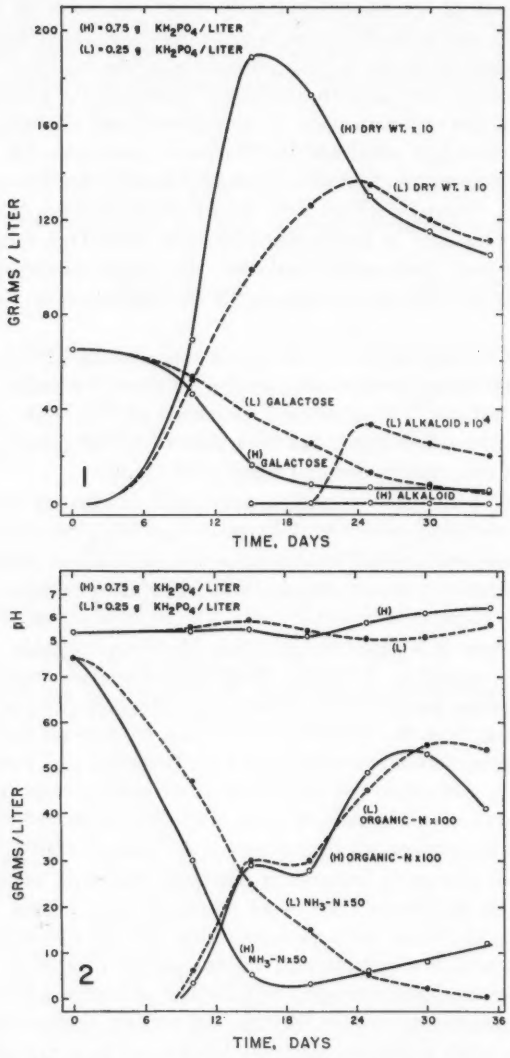
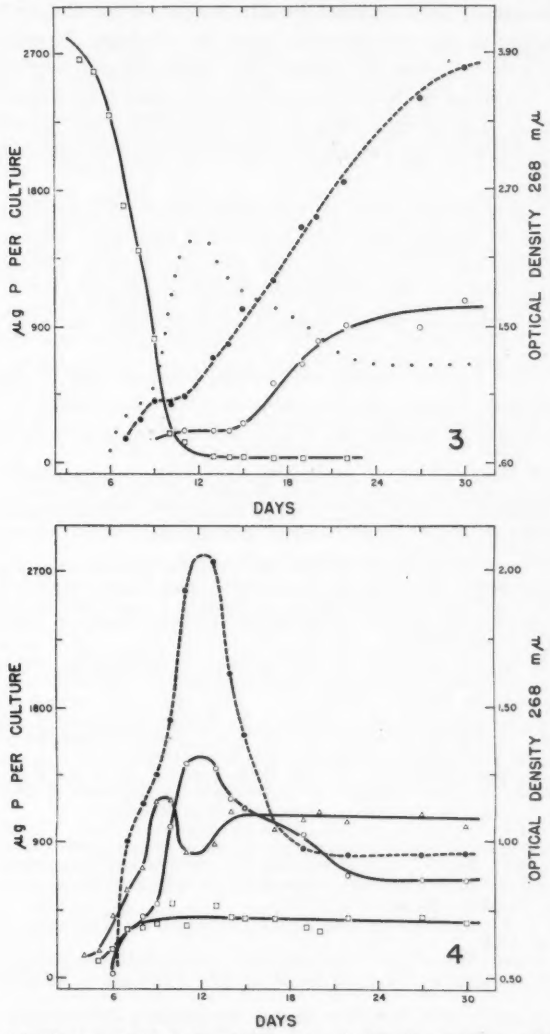
assay. Nucleic acids of both the TCA extracts and filtrate were estimated in a Beckman DU spectrophotometer at 268 mμ (6) and are reported in the figures as optical density per centimeter light path based on the original volume of the culture.




FIGS. 1 and 2. The course of growth, nutrient utilization, nitrogen excretion, pH, and ergot alkaloid production by shaken cultures differing in the initial orthophosphate concentration. Both the rate of growth and total growth is limited by the lowest concentration of phosphate.

Cultures grown under stationary conditions show (Figs. 3 and 4) that the inorganic phosphate was rapidly taken up from the medium and although some of the phosphate exists in the mycelium as inorganic, or highly labile




FIGS. 3 and 4. Changes in the form and quantity of phosphate compounds in the filtrate (Fig. 3) and in the mycelium (Fig. 4) of stationary cultures having an initial $KH_2PO_4$ concentration of 250 mg/liter.
— Phosphate expressed in μg phosphorus per flask. – – – Ultraviolet extinction coefficient based on the original volume . . Nucleic acids plus low-polymerized polyphosphates from Fig. 4. □ Orthophosphate. ●Nucleic acids. In Fig. 3 other absorbing organic material is included. ○ Nucleic acids plus low-polymerized polyphosphates calculated as total phosphorus minus orthophosphate phosphorus from TCA extract. △ Nucleic acids plus high-polymerized polyphosphates.

phosphate, most of the phosphate was rapidly converted to nucleic and high- and low-polymerized polyphosphates. If growth requires a continued supply of phosphate, some of the condensed or nucleic acid phosphate must have served as a phosphate reserve, since growth continued for almost 3 weeks after inorganic phosphate had disappeared from the medium. Noteworthy is the fact that the concentration of nucleic acid and low-polymerized polyphosphate of the mycelium at first increased rapidly and then began to decrease during the phase of prolonged, slow growth, and that their concentration was at a minimum prior to the period in which the ergot alkaloids are normally formed.

The more highly polymerized phosphate was partially depolymerized after its initial rapid formation and then attained a relatively high and constant level in the mycelium. This is consistent with the data of Krishnan (6). Particularly unexpected was the continual release of nucleic acid and polyphosphate into the medium during growth of mycelium starved partially of phosphate.

It is hoped that further studies may reveal whether this "leakage phenomenon" of phosphate-starved mycelium is in part responsible for placing the mycelium in such a state that the shunt metabolism products are formed.

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## TIME OF MOVEMENT OF POTATO LEAF-ROLL AND TURNIP-LATENT VIRUSES OUT OF LEAVES OF PHYSALIS FLORIDANA RYDB.[1]

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During studies on the transmission of the potato leaf-roll (LRV) and turnip-latent viruses (TLV) (4), both of which persist in their aphid vector, I have tested the time that these two viruses take to move out of inoculated leaves of *Physalis floridana* Rydb. Apterous aphids of *Myzus persicae* (Sulz.) were reared on detached leaves of *P. floridana* plants infected with one virus or the other. Large aphids from each colony were then caged singly on a leaf of a healthy *P. floridana* test plant in the six-leaf stage. After a feeding period of 6 or 24 hours on the test plants, the aphids were killed and the inoculated leaves removed at various intervals. After inoculation all the test plants were kept in a greenhouse for a month; the subsequent development of symptoms indicated those instances in which the virus had moved out of the inoculated leaves. As controls, the inoculated leaves were left intact on one group of representative plants.

The results of the first two experiments (Table I) show that neither virus moved out of inoculated leaves within 6 hours after being infested with viruliferous aphids, and LRV moved out of a few leaves within 24 hours. When inoculated leaves were removed at 48 hours, LRV had moved out of only two-thirds as many leaves as it did eventually in the controls, and TLV had not moved out of any. Since the level of transmission of TLV was low when aphids fed on test plants for 6 hours, the feeding time was increased to 24 hours in a third experiment. In this instance (Table I), TLV had moved out of a few leaves when they were removed 24 and 48 hours after the initial infestation, and in about one-half as many as in the controls when the inoculated leaves were removed after 72 hours.

In the foregoing experiments the distance from the inoculation site on the leaves to the main stem of the test plants was less than 5 cm, yet both these viruses usually took longer than 24 hours and in many cases longer than 48

TABLE I

Plants of *P. floridana* infected with LRV or TLV by single aphids when inoculated leaves were removed at intervals after infestation with aphids

| Virus | Feeding time on test plants, hr | Leaves removed after aphids put on, hr | | | | Uncut controls |
|---|---|---|---|---|---|---|
| | | 6 | 24 | 48 | 72 | |
| LRV | 6 | 0 | 2 | 34 | — | 50* |
| TLV | 6 | 0 | 0 | 0 | — | 10* |
| TLV | 24 | — | 1 | 4 | 10 | 21† |

*Plants infected out of 80.
†Plants infected out of 50.

[1]Contribution No. 38, Canada Department of Agriculture Research Station, Research Branch, Fredericton, New Brunswick.

hours to move this distance. Further evidence that LRV does not move rapidly through inoculated plants is the report by Bradley and Ganong (3) that it takes from 5 to 8 days for this virus to pass from aphids on the leaves of Katahdin potato plants into the developing tubers. Some mechanically inoculated viruses have been reported to take several days to move out of inoculated leaves (1), while certain leafhopper-transmitted viruses have been shown to move 20 cm or more from the site of inoculation within an hour (2, 5, 6). The accepted explanation is that the former must first pass through superficial tissues, while the latter are injected directly into the phloem. The rate of movement of LRV and TLV appears to resemble the mechanically inoculated viruses—this despite the fact that one of the viruses (LRV) is reputedly injected into the phloem (1). It would seem that either the accepted explanation for the difference in time required for movement or else the supposed site of inoculation of LRV (and TLV) should be reassessed.

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## STABLE OIL–AGAR EMULSIONS FOR USE IN CULTURE MEDIA

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Investigators studying the utilization of fats and oils by bacteria and fungi have encountered considerable difficulty in dispersing the fatty substances minutely and homogeneously in solid media for lipolytic studies. Any efficient and suitable method, therefore, for preparing such media would be of advantage.

Among various methods is the work of Goldman and Rayman (1), who have successfully prepared stable liquid emulsion culture broths with animal and vegetable oils, by means of a Waring blendor and a dairy homogenizer, for the purpose of quantitative analysis of lipolytic activity by microorganisms. These investigators have also devised a solid emulsion culture medium by adding agar, Nile blue sulphate, and other components to portions of their stock emulsion culture broth, for the isolation of lipolytic organisms.

In the course of working with *Hormodendrum resinae*, the following technique of achieving a stable and solid oil–agar emulsion was devised which is a relatively simple one giving efficient and reliable results for qualitative lipolytic studies. This method, not requiring the aid of emulsifying agents, involves the use of a portable hand homogenizer (Fig. 1). The hand homogenizer is made of stainless steel and aluminum alloy, and consists of a cup, cylinder, and hand-operated pump which forces the liquid to be homogenized through a small orifice with an approximate pressure of 600 pounds. The various parts of the homogenizer, as seen dismantled in Fig. 1, may be easily taken apart for cleaning.

The first step in preparing the solid oil–agar emulsion follows the usual method of sterilization of the agar culture medium, which is composed of the desired formula subject to sterilization, and which also includes the requisite amount of oil or other water-insoluble component not significantly altered by autoclaving. At the same time, the homogenizer, wrapped in brown paper, is autoclaved, together with a covered beaker to be used as a recipient vessel for the emulsion as it emerges from the pump.

After sterilization, in a transfer chamber, the warm and still liquid oil–agar solution is poured into the cup of the warm homogenizer. The homogenizer cup is kept protected from possible air contaminants by a cover fashioned of sterile aluminum foil or brown paper. Any materials to be included in the medium which should be sterilized by methods other than autoclaving may be incorporated into the mixture as it is poured into the cup.

The liquid solution is then pressed through the aperture of the homogenizer by means of the hand pump, and the emulsion collected in the sterile beaker. From the beaker, the warm, liquid emulsion may be either returned to its flask, or poured directly into sterile petri plates, where it rapidly cools and solidifies in a homogeneous phase. If the emulsified medium is to be stored in the flask, it may be gently reheated for pouring at a later time, without losing its homogeneity.

The homogeneous character of an oil–agar emulsion prepared in this manner will remain solid and stable at room temperature for a period of 1 to 2 months. Periods longer than 2 months do not alter the appearance of the medium, although shrinkage by water loss occurs in varying degrees, depending on the seal of the container.

There are but two main points of caution in the procedure. These are: first, the sterile mixture of oil, agar, and nutrients must be sufficiently warm, and thereby liquid, to give time for pressing it through the homogenizer and for subsequent pouring into plates, before it solidifies; second, the homogenizer itself should be as hot as can be comfortably handled in order that the emulsion may pass through the cylinder aperture without congealing in the bottom of the cup.

When emulsifying several mixtures of one series differing only in concentration of one of the components, it is convenient to place the flasks awaiting the processing in a pan of hot water. By emulsifying first the formula with the

lowest concentration of the given material, a series of 8 to 10 emulsions can be made in a short time without dismantling and cleaning the homogenizer and its parts.

Figure 2 shows fungus colonies of *Hormodendrum resinae* growing on various oil–agar substrates, and on glucose agar which, by comparison, appears transparent against the black background. The media represented in Fig. 2, used to demonstrate the lipolytic activity of *Hormodendrum resinae*, contain various oils, either cod liver oil, castor oil, corn oil, soybean oil, or linseed oil, at concentrations of 3%, each added to a basal Czapek mineral solution with 1.5% agar.

The use of the homogenizer technique is also well suited to the determination of lipase activity according to the method of Long and Hammer (2), which involves the incorporation of the simple triglyceride, tributyrin, in the agar medium. In testing the ability of deteriorative fungi to grow on creosote, the homogenizer has proved equally helpful in achieving a finely dispersed stable emulsion.

In view of the ease and success encountered with this method in preparing various culture media containing oils, fatty acids, or creosote, it should prove useful and adaptable wherever there is need for stable emulsions of water-insoluble compounds in a basic agar medium.

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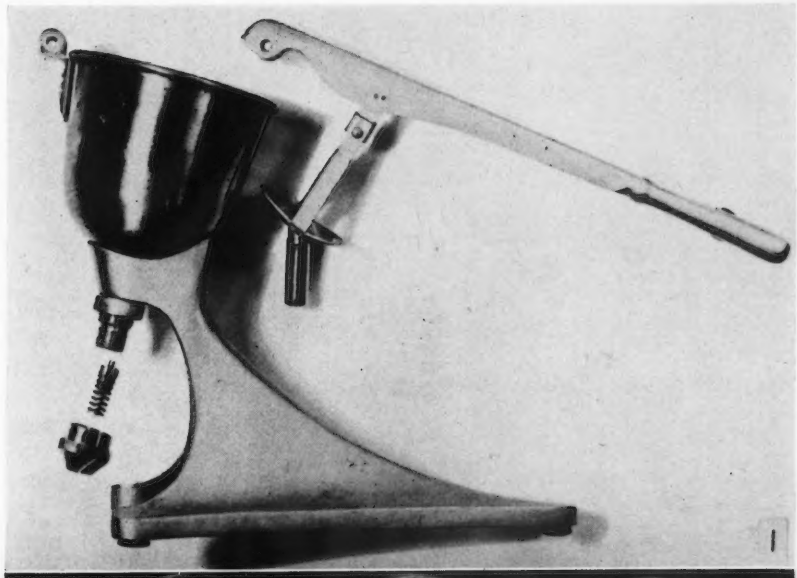


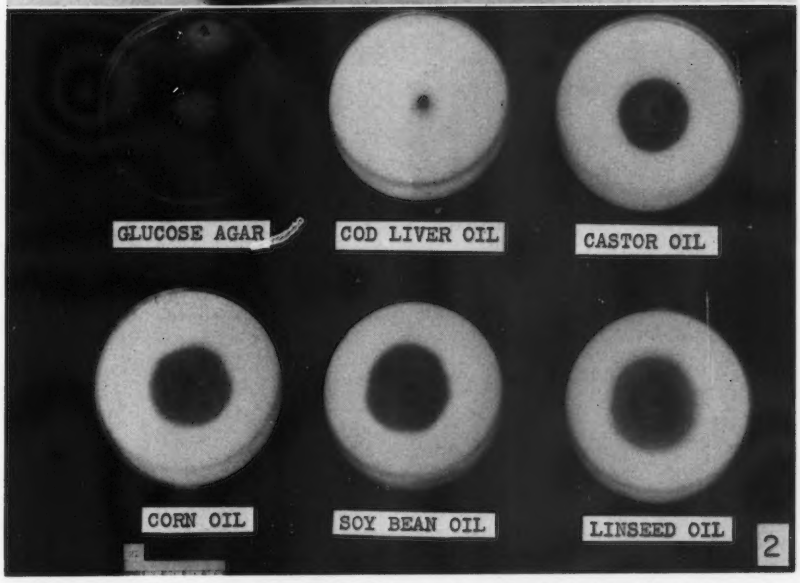


FIG. 1. Exploded view of portable hand homogenizer. (Available from Central Scientific Co. Catalogue No. 70180.)

FIG. 2. *Hormodendrum resinae* colonies on various oil–agar substrates, and on glucose agar. Top row, left to right: glucose agar, cod liver oil agar, castor oil agar. Bottom row, left to right: corn oil agar, soybean oil agar, linseed oil agar. Note the white opaque appearance of the oil–agar media, as compared with the translucence of glucose agar.

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